UNIVERSITY OF EDINBURGH

INVESTIGATIONS OF THE POLYSACCHARIDES FROM THE GUM EXUDATE OF BRACHYCHITON DIVERSIFOLIUM

by

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A Thesis for the Degree of Doctor of Philosophy

August, 1957.

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INTRODUCTION

INTRODUCTION

Plant gums have been adequately described by Jones and Smith (1) as "substances of plant origin which are obtained as an exudation from the fruit, trunks or branches of trees spontaneously or after mechanical injury of the plant by incision of the bark, or by removal of a branch, or after invasion by bacteria or fungi". In contact with water these gums have the outstanding ability of form-:ing gelatinous pastes or highly viscous solutions. Plant mucilages also have this property which is due in both instances to the characteristic polysaccharide constituents. Since, in addition, both types of polysaccharide have similar molecular structures, they are generally reviewed together (1-7). They are both polyuronides; D-glucuronic acid residues are generally found in gums and D-galacturonic acid residues in mucilages, though this is not an invariable rule. In addition, hexoses, pentoses and methylpentoses have been isolated from different gum hydrolysates.

A primary distinction between gums and mucilages lies in their mode of formation, for whilst gums exude and dry, leaving discrete nodules or ribbons which may be removed mechan-:ically from the site of origin, mucilages form

a protective covering for different plant organs and can only be isolated by hot or cold water extraction. Examples of the latter are the galactomannans of the guar (8) and carob (9) seeds and it is important to note that these polysaccharides are nevertheless known as "gums".

The discussion to follow will, however, be confined to polyuronide exudations.

Whilst gum exudation has one invariable effect, namely that of sealing-off the damaged or invaded area, a variety of reasons has been advanced to account for gum-formation. It seems unlikely that there is one universal explanation but rather that the factors affecting gum format-:ion may vary from one specific instance to another.

A particular aspect of the subject which is imperfectly understood is the extent to which micro-organisms are active in producing or inducing the formation of exudate on gum-producing trees, and whether the gum itself is formed to prevent such a bacterial infection or is a result of that infection. Whilst it is conceivable that the structure of the gum could depend on the type of invading micro-organism, varying perhaps

from one site on the tree to another and irrespective of botanical classification, the converse has been indicated in certain investigations. Two types of cherry tree have been found to produce gums of markedly similar compositions (42) though differing from that examined by earlier workers (97), whilst the gums from several damson trees (23) had substantially the same constants. In addition, gum tragacanth exudes so soon after rupture of the bark, that it is considered unlikely to be an insect or bacterial product.

It has also been suggested, however, that gum exudation by a tree represents a reaction to conditions of drought denoting also, perhaps, that the tree is in poor health. In agreement with the first suggestion is the fact that commercial sources of gum arabic are invariably trees in hot dry climates, whilst in support of the latter argument are statements that some gums, honey locust gum (119) for example, are definitely pathological products.

The origin of plant gums is uncertain, but is believed by some authorities to be the starch granules present in the cells. Whilst the gums are worthy of investigation in their own right and as an aspect of plant biochemistry, additional

interest derives from their structural similarity to the bacterial polysaccharides.

Relatively few in number of the known plant gums find commercial applications, but certain of them, for example gum arabic and gum tragacanth are used in immense quantity and are very important items of trade.

In foodstuffs (48) gums are particularly suited, by virtue of their high viscosity in solution and inability to crystallise, to serve as thickeners for beverages, as stabilisers for oil and water emulsions and, as a wider application of this latter use, as protective colloids whose function it is to prevent agglomeration and settling-out of minute particles. These properties also make gums of wide application in the preparation of pharma-:ceuticals, though in this connection uncertainty about their suitability arises from a recent claim (120) that gum tragacanth and probably other gums exert a strong neutralising effect on the activity of certain bactericides normally added as preservatives. In the textile industries gums are widely used to impart lustre to certain materials, for example silk, and as

thickeners for colours and mordants in calico-:printing.

Gums may moreover serve as sources of monosaccharides, thus mesquite gum serves as a convenient source of L-arabinose which can be dialysed out of a neutralised hydrolysate and crystallised by addition of ethanol (49), or alternatively, isolated by crystallisation from methanol after removal of acidic oligosaccharides on ion exchange resins (50) or as precipitated barium salts (51). Gum arabic has been advocat-:ed as a source of D-glucuronic acid (52).

Purification and Properties.

The nodules of gum contain polysaccharide material contaminated with "mechanical" impurities such as bark fragments, entrapped dust and insects. In addition enzymes, inert proteinaceous material and varying amounts of terpenoid resins can also be present.

The polysaccharides are of complex structure. The uronic acid residues may carry acetyl or methyl groups and generally occur at least in part as the metallic salts. Hexose residues are present in the pyranose configuration whilst

pentose residues widely occur in the furanose form. Recent work (11, 13, 14, 15, 87, 125) has revealed the presence of pentose pyranosides in plant gums.

A summary of the constituents which have been established as belonging to plant gums is given in Table I.

TABLE I

Plant Gum	Uronic Acid	Hexose	Pentose	Methyl pentose	Ref.
Phormium tenax	D-Glu		D-Xy		37
Honey Locust (Gleditschia triacanthos)	D-Glu	D-Ga	L-Ar		119
Silk Oak (Grevillea robusta)	D-Glu	D-Ga	L-Ar		39
Arabic (Kordofan)	D-Glu	D-Ga	L-Ar	L-Rh	19
Black Wattle (Acacia mollissima)	D-Glu	D-Ga	L-Ar	L-Rh	20
Acacia pycnantha	D-Glu	D-Ga	L-Ar	L-Rh	17
Acacia cyanophylla	D-Glu	D-Ga	L-Ar	L-Rh	16
Acacia karoo	D-Glu	D-Ga	L-Ar	L-Rh	11
Egg Plum	D-Glu	D-Ga	L-Ar, D-Xy		24
Almond	D-Glu	D-Ga	L-Ar, D-Xy		18
Chagual (Puya sp.)	D-Glu	D-Ga	L-Ar, D-Xy		123
Peach (Prunus persica)	D-Glu	D-Ga	L-Ar, D-Xy	L-Rh	36
Neem (Melia azadirachta)	D-Glu	D-Ga	L-Ar, D-Xy	L-Fu	34
Cherry	D-Glu	D-Ga, D-Ma	L-Ar, D-Xy		21
Damson	D-Glu	D-Ga, D-Ma	L-Ar, D-Xy		23
Hakea acicularis	D-Glu	D-Ga, D-Ma	L-Ar, D-Xy		28
Ghatti (Anogeissus latifolia)	D-Glu	D-Ga, D-Ma	L-Ar, D-Xy	L-Rh	26

TABLE I (Contd.)

Plant Gum	Uronic Acid	Hexose	Pentose	Methyl Pentose	Other Sugars	Ref.
Mesquite (*Prospis juliflora*)	4-Me-D-Glu	D-Ga	L-Ar			31
Myrrh (*Commiphora myrrha*)	4-Me-D-Glu	D-Ga	L-Ar			33
Lemon	4-Me-D-Glu	D-Ga	L-Ar			15
Grapefruit	4-Me-D-Glu	D-Ga	L-Ar			27
Frankincense (*Boswellia carterii*)	4-Me-D-Glu	D-Ga	L-Ar			25
Modal (*Lannea grandis*)	4-Me-D-Glu	D-Ga	L-Ar			32
Sapote	Me-D-Glu		L-Ar, D-Xy			38
Sterculia setigera	D-Gal	D-Ga		L-Rh	D-Ta	40
Sterculia urens	D-Gal	D-Ga		L-Rh		104
Cochlospermum gossypium	D-Gal	D-Ga		L-Rh	D-Ta	29
Cholla (*Opuntia fulgida*)	D-Gal	D-Ga	L-Ar, D-Xy	L-Rh		22
Tragacanth (*Astralagus*)	D-Gal	D-Ga	D-Xy	L-Fu		100
Khaya grandifolia	D-Gal 4-Me-D-Glu	D-Ga		L-Rh		30

KEY:

D-Glu:- D-glucuronic acid;
4-Me-D-Glu:- 4-O-methyl-D-glucuronic;
D-Gal:- D-galacturonic;
D-Ga:- D-galactose; L-Ar:- L-arabinose;
D-Ma:- D-mannose; D-Xy:- D-xylose;
L-Rh:- L-rhamnose; L-Fu :- L-fucose;
D-Ta:- D-tagatose.

In addition to the foregoing, the following gums have been examined:-

The gum component of Olibanum (*Boswellia carteri*) is composed of an arabo-galactan and a polysaccharide containing galactose and galacturonic acid (35).

Golden apple gum (*Spondias cytheria*) contains galactose, arabinose, xylose, glucuronic acid (mainly, if not entirely, as mono-*O*-methyl ether) and traces of rhamnose and fucose (14).

Khaya senegalensis gum contains galactose, rhamnose, and probably 4-*O*-methyl-D-glucuronic acid and galacturonic acid (30).

Sterculia tormentosa gum contains rhamnose, galactose and probably galacturonic acid (102).

Chromatographic evidence has been obtained for the compositions of the following:-

Ketha gum (*Feronia elephantum*) - uronic acid, galactose, arabinose, xylose and rhamnose (122).

Anogeissus schimperi gum - glucuronic acid, galactose and arabinose (37).

No detailed comparisons have been drawn between the structures of gums from different

species of trees, but Jones and his colleagues (13) believe that D-galactose and uronic acid residues generally constitute the backbone of gum polysaccharides with 1-3 and 1-6 linkages predominating, whilst side chains are characterised by the presence of D-xylopyranose, L-arabopyranose and L-arabofuranose linkages.

Isolation of the pure polysaccharide from the crude gum involves dissolution of the specimen in water or in dilute sodium hydroxide solution. The "free-acid" polysaccharide can then be obtained by use of cation exchange resins (45) or more generally by precipitation in glacial acetic acid (43) or acidified methanol or ethanol. Purification of the polysaccharide is effected by repeated solution in water and reprecipitation, or electrodialysis (44) can be used.

Homogeneity of the Polysaccharide.

There is no guarantee that the material isolated in this manner is a single polysaccharide and fractionation of several plant gums has in

fact been achieved.

Chemical fractionation can be attempted by graded precipitation, for example by the calibrat-:ed addition of ethanol to an aqueous solution of the polysaccharide. As a result, a number of precipitates corresponding to specific alcohol: water ratios are isolated and can be examined for optical rotation and constituent sugars. Since, however, this type of solvent fractionation also serves as a method of separating specific polymers into fractions of approximately equal chain length, the isolation of more than one fraction is not by itself an indication that more than one poly-:saccharide is present.

In addition to the above, chance solvent effects can also effect fractionation. The gum component of Olibanum (35) was fractionated to yield a galactoaraban and a polyuronide composed of galactose and galacturonic acid by the addition of ethanol and subsequently acetone to an aqueous solution of the gum. Gum tragacanth (100) was also fractionated, but not until after methylation when a mixture of an acidic methylated poly-:saccharide, a neutral methylated polysaccharide and a glycoside was obtained. The last of these was removed by treatment with cold water in which

it alone was insoluble. On heating the aqueous solution the neutral methylated polysaccharide was precipitated, the acidic polysaccharide (as sodium salt) remaining in solution.

Recent work on gum arabic (47) has shown that immunological fractionation can prove a useful tool in the separation of polysaccharides. In this instance the polysaccharide recovered from its specific precipitate with Type II anti-:pneumococcus serum contained only one-third to one-fifth as much rhamnose as the native gum though the composition with respect to the other monosaccharides in the gum was not significantly altered. Several attempts at fractionation using iso-propyl alcohol or acetic acid were less successful (47).

Electrophoresis of solutions of poly-:saccharides in a Tiselius apparatus offers some criterion of homogeneity. Gum arabic and cyanophylla gum have been found to possess different mobilities (53), whilst Jones and Pridham (54) have also resolved a mixture of gum arabic and a plum gum by this method. It has been found, however, by Colvin, Cook and Adams that the electrophoretic technique employing

polysaccharide solutions in molar potassium hydroxide solution is incapable of resolving certain mixtures of structurally different polysaccharides (55). Such a mixture as inulin, wheat starch and sodium alginate for example failed to resolve, and the opinion of these authors is that contrary to previous reports the technique cannot be used with confidence as a general criterion of the purity of polysaccharides, but might be applicable to the characterisation and fractionation of a limited class of polysaccharides which are soluble close to neutrality.

Preliminary Examination.

In a preliminary investigation of the purified material, the determination of ash con-:tent reveals the presence of persisting inorganic impurity, whilst methoxyl content of an alcohol-:free polysaccharide denotes methyl ether or ester. The latter group is relatively labile, however, and is often hydrolysed during the purification of the polysaccharide. A decrease in methoxyl content corresponding to an increase in titratable acidity has been shown to occur on hydrolysis of

gum tragacanth (56) indicating esters. Acetyl groups are also relatively labile and are estimat-:ed by hydrolysis followed by distillation and titration of the liberated acetic acid (57). Free carbonyl groups are determined by oxidation with alkaline iodine solutions (58), whilst the proportion of uronic acid can be estimated by direct titration since "free-acid" polyuronides are comparatively strong acids - arabic acid for example gives solutions of pH 2.2-2.7; a conventional acid-base colour indicator can be used or a potentiometric method, preferably in presence of sodium bromide (93). Uronic acids are also susceptible to decarboxylation under acid conditions, and this may be employed quantitatively, the carbon dioxide evolved being measured manometrically (59) or by absorption in standard alkali (60).

Hydrolysis and Paper Chromatography.

Considerable information concerning the structure of the polyuronide can be obtained from hydrolytic experiments. The glycosidic linkage of a furanose residue is ruptured under relatively

mild conditions, for example by heating an aqueous solution of the "free-acid" polysaccharide, a process known as autohydrolysis. This gives information generally applying to the periphery of the molecule and offers a means of obtaining a "degraded" or partly-simplified polysaccharide for methylation studies; oligosaccharides can also be obtained. Linkages between neutral pyranoside residues are less easily hydrolysed than furanos-:ides, whilst a uronic acid in glycosidic union with a neutral sugar forms an extremely stable bond; this last consideration means that complete hydrolysis of a polyuronide requires relatively concentrated mineral acid at elevated temperatures and results in considerable degradation of the material.

The neutralised hydrolysate may be analysed for neutral sugars on the paper chromatogram by some variation of the method introduced into carbohydrate chemistry by Partridge (61). A wide variety of solvents having special character-:istics is available, but for general investig-:ations systems based on n-butanol (62) or ethyl acetate (63) are suitable. The former solvent has the advantage that a comprehensive list of R_G values (the ratio of the distance

travelled by the sugar to that travelled by tetramethylglucopyranose under the same conditions) is available (64). The employment of specialised conditions, for example the use of a borate-:saturated system (65) may serve to effect or enhance specific resolutions because of the ability of the borate ion to complex with adjacent *cis* hydroxyl groups of certain sugars, which has a marked effect on their chromatographic mobility.

Under the above conditions, lactones of uronic acids will also be detected; D-glucurone for example runs slightly faster than L-rhamnose. In general, however, the uronic acids are present in the neutralised hydrolysate as salts remaining on or near the starting-line of the paper chromatogram. After removal of cations the free acids may be chromatographically examined in a solvent containing formic or acetic acid; the former has been recommended (67) as yielding more reproducible results. Resolution of uronic acids has also been achieved on paper buffered with alginic acid (68).

Revelation of the separated hydrolysis products is achieved by the application of a suitable spray reagent. A wide variety of these

is available, but ammoniacal silver nitrate solution (69) or an alkaline mixture of periodate and permanganate (70) is highly suited to general detection. Specific sprays for the detection of particular functional groups are also available; urea hydrochloride, for example, reacts characteristically with ketoses (71), whilst acid:base indicator sprays may be used to reveal uronic acids (72). The use of the above techniques coupled with that of paper electrophoresis of the negatively-charged borate complexes of the sugars (95) enables tentative identification of the hydrolysis products to be made.

The chromatographic procedure can be extended to the estimation of the relative proportions of the monosaccharides. A widely-used modification of this technique is that of spraying "guide-strips" from each side of the developed chromatogram. By this means, bands of monosaccharides are are located on the unsprayed paper, eluted and estimated by a suitable micromethod, for example with the Somogyi copper reagent (73). An alternative technique is that of spraying a normal type of chromatogram with p-anisidine hydrochloride and heating. The spots

obtained are cut out and eluted, and the absorbanc-:es of the solutions determined spectrophoto-:metrically then related to the weight of sugar present by reference to calibration curves (75).

Separation and Characterisation of Hydrolysis Products.

Autohydrolysis or partial acid hydrolysis of a polyuronide may yield a mixture of neutral mono-:saccharides and oligosaccharides as well as aldo-:biuronic acids; the latter can then be conven-:iently separated as barium salts by alcohol-:precipitation after neutralisation. The mixture of neutral sugars and oligosaccharides can be separated into groups of similar molecular weight by passing through carbon columns (76). Mono-:saccharides are eluted with water, and the oligo-:saccharides fractionated by elution with gradually-increasing concentrations of ethanol in water.

Separation of monosaccharides on the preparat-:ive scale has been widely achieved by partition chromatography on columns of cellulose (77), a method which has the advantages of the high resolving powers and working capacities of

cellulose coupled with the direct comparison of behaviour of monosaccharides on these columns and on the paper chromatogram.

Columns of Celite (78) exhibit behaviour similar to that of cellulose, whilst potato starch columns (79) have excellent powers of resolution, but suffer from low working capacity. Khym and Zill (80) have fractionated sugar mixtures as borate complexes on columns of ion exchange resin, but have not applied this technique on the preparative scale.

Whilst the monosaccharides are characterised as crystalline compounds or derivatives, the elucidation of the structures of oligosaccharides and aldobiuronic acids is achieved by methylation, hydrolysis and identification of the products. The difficulty raised by the resistance to hydrolysis of the fully-methylated acidic disaccharides can be overcome by reduction with lithium aluminium hydride (10); by this means neutral methylated disaccharides are obtained which can then be easily hydrolysed.

A number of neutral and acidic disaccharides have been obtained from gums; details are given overleaf.

Neutral Disaccharides.

(i) 3-O-β-L-Arabopyranosyl-L-arabinose has been isolated from peach (13), cherry (13), golden apple (14), lemon (15), and Acacia karroo (11) gums.

(ii) 5-O-α-L-Arabopyranosyl-L-arabinose has been isolated from Virgilia oroboides gum (125).

(iii) (4- or) 5-O-β-D-Xylopyranosyl-L-arabinose has been isolated from peach and cholla gums (13).

(iv) 3-O-α-D-Xylopyranosyl-L-arabinose has been isolated from golden apple gum (14).

(v) 3-O-D-Galactopyranosyl-L-arabinose has been isolated from arabic (19) and Acacia cyanophylla (16) gums.

Aldobiuronic Acids

(i) 6-O-D-Glucuronopyranosyl-D-galactose has been isolated from Acacia cyanophylla (16), Acacia karroo (11), Acacia pycnantha (17), Acacia mollissima (20), arabic (90), almond (18), egg plum (24), ghatti (26) and peach (36) gums.

Aldobiuronic Acids (Contd.)

(ii) 6-O-(4-O-Methyl-D-glucuronosyl)-D-galactose has been isolated from mesquite (92) and modal (46) gums.

(iii) 4-O-D-Glucuronosyl-D-galactose has been isolated from Acacia karroo (11) and neem (34) gums.

(iv) 4-O-(4-O-Methyl-D-glucuronosyl)-D-galactose has been isolated from Khaya grandifolia (30) and myrrh (115) gums.

(v) 4-O-(4-O-Methyl-D-glucuronosyl)-L-arabinose has been isolated from lemon gum (12).

(vi) 2-O-D-Glucuronosyl-D-mannose has been isolated from damson (23), ghatti (26), Hakea acicularis (28) and cherry (21) gums.

(vii) 2-O-D-Glucuronosyl-D-xylose has been isolated from chagual gum (123).

(viii) 4-O-D-Galacturonosyl-D-galactose has been isolated from Cochlospermum gossypium (29) and Sterculia setigera (40) gum.

(ix) 2-O-D-Galacturonosyl-L-rhamnose has been isolated from Cochlospermum gossypium (29), Sterculia setigera (40) and Khaya grandifolia (30) gums.

In addition to the above, an aldobiuronic

acid has been isolated from lemon and grapefruit gums (27) in which D-glucuronic acid or a methyl-:ated derivative was linked to the 4 position of D-galactose. Mesquite gum has been found by White (31) to contain an aldobiuronic acid in which 4-O-methyl-D-glucuronic acid is glycosidically joined to position 3 of D-galactose, whilst Cuneen and Smith (91) report the isolation from this gum of two aldobiuronic acids comprising the same structural units linked 1-4 and 1-6 respectively.

Methylation Studies.

The greatest contribution to the elucidation of the structures of plant gums, as for other polysaccharides, has come from the technique of methylation, hydrolysis and identification of the methylated monosaccharides produced.

Methylation is generally achieved by treat-:ment of the polysaccharide with dimethyl sulphate and sodium hydroxide solution (81). Successive applications of the reagents are made until the methoxyl content is constant; this can often then be raised by Purdie methylation by heating with silver oxide and methyl iodide under

anhydrous conditions (82).

Where resistance to aqueous methylation is encountered, thallous hydroxide (83) or ethoxide form useful alternatives and have been used to methylate cherry (130), damson (127) and egg plum (124) gums.

Phormium tenax gum (37) has proved resistant to both of the above methylation techniques, but might conceivably be methylated by treatment with metallic sodium in liquid ammonia and then with methyl iodide (103).

Hydrolysis of a methylated polysaccharide with hot aqueous mineral acid is not practicable since under these conditions the polysaccharide will precipitate from solution and merely degrade as opposed to hydrolysing. This can be overcome, however, by allowing the methylated polysaccharide to stand in relatively concentrated acid for an extended time at room temperature, the hydrolysis being completed by heating. An alternative and more general procedure is to heat with anhydrous methanolic hydrogen chloride either under reflux or in a sealed tube, producing methylated methyl glycosides. Traditionally, these products were isolated and examined, after fractional distillat-

:ion under high vacuum. This technique has been superseded by chromatographic methods. Generally, the glycosides are hydrolysed and the free methyl-:ated sugars separated by partition chromatography on columns of cellulose. Bell (86) has resolved mixtures of methylated derivatives of glucose on silica gel and has extended this technique to the resolution of methylated derivatives of fructose. Whelan and Morgan (88) have shown that methylated derivatives of glucose can be separat-:ed on charcoal using increasing concentrations of ethanol in water as eluant, whilst White (87) has successfully resolved the mixture of methylat-:ed sugars from sapote gum using columns of acid-:washed Magnesol and an extrusion technique.

The following methylated monosaccharides have been isolated from methylated plant gums; the figures in the columns refer to the positions at which the methyl groups are attached to the sugars.

Methylated Plant Gum	D-Glu	D-Ga	L-Ar	D-Xy	L-Rh	L-Fu	D-Gal
Arabic (128)	2:3:4 2:3	2:3:4:6 2:4	2:3:5 2:5		2:3:4		
Cherry (130)	2:3:4 2:3	2:4:6 2:4	2:3:5 2:5				
Damson (127)	2:3:4 2:3	2:4:6 2:4 4? 2	2:3:5 2:3				
Egg plum (124)	2:3	2:4:6 2:4	2:3:5 2:5 2	2:3:4			
Mesquite (129)	2:3:4	2:4	2:3:5 3:5				
Sapote (87)	2:3:4 3:4		2:3:4	2:3:4 3			
Sterculia setigera (41)		2:3:4:6 2:3:6 2:6			3:4 2 and/or 3		2
Cholla (22)		2:4	2:3:5 2:3	2:3:4			2
Tragacanth (100)				2:3:4 3:4		2:3:4	2:3 mono
Khaya grandifolia (30)	2:3:4	2:3:4:6 2:3:6			3		2:3

Key:

D-Glu:- D-glucuronic acid
D-Gal:- D-galacturonic acid

D-Ga:- D-galactose
L-Ar:- L-arabinose
D-Xy:- D-xylose
L-Rh:- L-rhamnose
L-Fu:- L-fucose

By the employment of the techniques already described, some indication of the structures of a number of gums has been obtained. Although it has not proved possible to establish a unique structure for any gum, formulae have been advanced for arabic (66) and mesquite (89) gums conform-:ing with the experimental facts.

Through the kindness of Mr. V. Grenning, Director of Forests in Queensland, Australia, a sample of gum from Brachychiton diversifolium collected in the Atherton district of Northern Queensland was made available for study.

Although the botanical name suggests that this gum has no connection with any already examined, this is not the case, for the term Brachychiton defines certain trees formerly known as Sterculia; Brachychiton diversifolium was previously known as Sterculia caudata. It is of interest, therefore, to review the previous studies of the Sterculia gums before describing the results of a structural investigation of the gum from Brachychiton diversifolium.

Sterculia tormentosa gum.

This gum has received some attention from Beauquesne (102) who found it much less susceptible to hydrolysis than arabic or tragacanth gums. Enzymic hydrolysis was unsuccessful, but acid hydrolysis was achieved by heating with 4% sulphuric acid for 20 hours at 100°, followed by 6 hours at 116°.

Galactose (43%) was identified by oxidation of uronic acid-free fractions of the hydrolysate to mucic acid. The osazone and α-methylphenyl

hydrazone were also prepared. Rhamnose (15%) was characterised as the methylphloroglucide and the osazone, whilst the uronic acid (43% of the gum) gave a brick red precipitate with basic lead acetate, suggesting galacturonic acid.

Sterculia setigera gum (40, 41).

The crude gum was found in a partly-acetylated condition (acetyl content: 15.5%) and yielded on purification an acetyl-free polysaccharide of equivalent weight 370-400.

Hydrolysis (to which the polysaccharide was very resistant) and quantitative chromatography showed that galactose and rhamnose were present in equimolecular amounts, whilst traces of rhamnoketose and tagatose were also evident.

Mild acid hydrolysis gave a mixture of sugars and a stable acidic fraction, the latter being isolated by precipitation as barium salts in methanol. The non-acidic reducing sugars were analysed by quantitative paper chromatography and shown to be constituted as follows: galactose (7 parts), tagatose (2 parts), rhamnose (5 parts) and rhamnoketose (traces). Separation of the mixture on a column of cellulose gave D-galactose, D-tagatose and L-rhamnose which were characterised,

whilst traces of rhamnoketose and xylose were also indicated.

Part of the acidic fraction was completely hydrolysed and D-galacturonic acid identified as a product, whilst the remainder was methylated. The behaviour of the fully-methylated product suggested that a biuronoside (1 part) and an aldobiuronic acid (2 parts) were present. Hydrolysis gave the neutral sugars 3:4-di-O-methyl-:L-rhamnose (86%) and 2:3:6-tri-O-methyl-D-:galactose (14%). The acidic fraction was mainly 2:3:4-tri-O-methyl-D-galacturonic acid and (3:4-) or 2:4-di-O-methyl-D-galacturonic acid. On this evidence it was deduced that the following had been produced by partial hydrolysis of the gum:

(i) 2-O-D-galacturonosyl-L-rhamnose

(ii) (3- or) 2-O-D-galacturonosyl-D-galacturonic acid

(iii) 4-O-D-galacturonosyl-D-galactose.

The gum was methylated and hydrolysed when a mixture of neutral and acidic reducing sugars was obtained. The former consisted of:

2:3:4:6-tetra-O-methyl-D-galactose (6 parts)

2:3:6-tri-O-methyl-D-galactose (3 parts)

2:6-di-O-methyl-D-galactose (trace)

Also present were methylated derivatives of rhamnose probably:

3:4-di-O-methyl-L-rhamnose (4 parts)

2- and/or 3-O-methyl-L-rhamnose (3 parts)

The methylated uronic acid was predominantly 2-O-methyl-D-galacturonic acid (16 parts).

It was deduced that the gum contained the following residues linked at the positions indicated:

D-Ga.1- -4.D-Ga.1- -2.L-Rh.1-

-4.L-Rh.1- and/or -4.L-Rh.1- -4.D-Gal.1-
2 3 3

The presence of large amounts of end groups and of triply-linked galacturonic acid showed the highly-branched nature of the macro-molecule.

Sterculia urens gum (104).

In a preliminary study on the exudate from Sterculia urens acetyl groups (6.72%) were again found, and D-galactose and L-rhamnose were identified; no tagatose was detected. The relative molecular proportions of galactose and rhamnose (6:4) were not significantly different from those found in Sterculia setigera and again

a high galacturonic acid content (37.4%) was recorded. Partial hydrolysis gave rise to what might have been two aldobiuronic acids: a galacturonosyl-D-galactose and a galacturonosyl-L-rhamnose, but these were not further investigated.

In brief, then, gum exudates of the Sterculia trees have been found to be polymers of rhamnose, galactose and galacturonic acid residues, partly acetylated in two instances at least. Sterculia setigera alone contains tagatose, but all three are characterised by a high uronic acid content and are consequently very resistant to hydrolysis.

D I S C U S S I O N

DISCUSSION

The Crude Gum.

The gum was relatively free from bark and consisted of brittle nodules of varying size having a resinous odour. In contact with water it swelled and formed a stiff gelatinous mucilage even at relatively low concentration. Investigation of three dissimilar nodules indicated that the sample was essentially homogeneous.

Analysis showed the presence of ash (4.1%), due at least in part to metallic salts of the uronic acid; the mucilage was neutral to litmus. A small amount of methoxyl group (1.4%) was present signifying methyl ether or methyl ester. The latter group was verified by its lability to alkali, since the purified polysaccharide, which was isolated after alkaline treatment of the gum, was free from methoxyl groups. Methyl ester has not been reported in the other *Sterculia* gums, but has been found in gum tragacanth (56). Acetyl groups (19.3%) were found approximating in proportion to those in *Sterculia setigera* (15.5%) and *Cochlospermum gossypium* (18.9%) (29) gums and considerably higher than those in *Sterculia tormentosa* (6.7%).

The gum was non-reducing to Fehling's solution

and did not form an insoluble copper complex; it was also free from protein.

Hydrolysis and chromatography of the hydro-:lysate gave evidence, which was fully confirmed in later work, that the gum was composed of:

L-rhamnose, D-galactose and D-glucuronic acid.

The gum from Brachychiton diversifolium (syn. Sterculia caudata) is thus different from the gums so far investigated, though certain resemblances are apparent. In the first place, it contains the same neutral sugars as the other Sterculia gums, but differs from them and approx-:imates more truly to the "gum" classification in containing D-glucuronic acid. Khaya grandifolia gum (30) offers a closer comparison since it contains the same neutral sugars together with 4-O-methyl-D-glucuronic acid as well as D-galacturonic acid.

Traces of pentoses (possibly xylose and arabinose) were also present in the crude gum and persisted in the pure polysaccharide, in which they were detected, however, only in large-scale work and not by quantitative chromatography. The proportions present, however, were so low that they can have very little structural significance and can probably be attributed to other botanical

residues present. It was noticeable that bark-contaminated gum gave relatively more pentoses than did the purer samples.

The gum was slightly soluble in water, from which it was best precipitated with acetone. Three fractions were obtained of essentially identical composition. Further attempts at fractionation by dissolution of the gum in alkali then precipitation of the "free-acid" form by addition of ethanol were equally unsuccessful. These results are in harmony with the electro-:phoretic behaviour of the pure polysaccharide in a borate buffer of pH 10, when a single peak was obtained. By contrast, however, Smith (126) reports that the polysaccharide on glass paper electrophoresis exhibits two components having mobilities of 7 and 108 (relative to the fastest moving component of ox-liver glycogen), the former being the major component; the precise signific-:ance of these results has not, however, been defined.

Purification and Properties of the Polysaccharide.

The gum was treated with aqueous sodium hydroxide solution, the mixture acidified and the

polysaccharide precipitated in ethanol. After redissolving in water and a further acidic precipitation, the product was freed from chloride ions to yield the pure polysaccharide (55% by weight of the crude undried gum) having $[\alpha]_D^{17}$ + 69°; ash 0.83%; acetyl nil. The positive rotation of the polysaccharide and methylated polysaccharide and the fall in rotation on acid hydrolysis indicates the presence of α-linkages. After purification in acetic acid to remove adhering ethanol, methoxyl was found to be absent.

The proportion of uronic acid present was estimated by two methods; firstly, by direct titration which gave an equivalent weight of 342 and, secondly, by thermal decarboxylation which gave a uronic anhydride content of 50.1%. These results are substantially in agreement since an equivalent weight of 342 requires a uronic anhydride content of 51.5%. The proportion of uronic anhydride found is unusually high for a plant gum, but finds parallels in Sterculia setigera gum (equivalent weight 370-400) (40) and Khaya grandifolia gum (30) (equivalent weight 344).

In keeping with the high proportion of uronic acid present and the extreme stability of the glycosiduronic acid linkage, the polysaccharide proved to be very resistant to hydrolysis. On autohydrolysis an iodine number of only 8.1 was obtained after 24 hours heating at 100°. Hydrolysis with N-sulphuric acid at 100° for 6 hours gave an iodine number of 51 and a change in specific rotation from +104° (2 hours) to +91°. Further heating for one or two hours produced little change, suggesting that the residual linkages were resistant to hydrolysis under these conditions and that further acid treatment would cause degradation rather than hydrolysis. In agreement with this, the iodine numbers of the solution became erratic on further heating. Complete hydrolysis of the polysaccharide was only achieved with 2N-sulphuric acid after heating for 24 hours at 100°. This was accompanied by degradation, since the solutions invariably darkened during vigorous hydrolysis and the yield of hydrolysate was never higher than 80%.

Although a complete quantitative hydrolysis was impossible, the relative proportions of the neutral sugars in the hydrolysate obtained after

complete hydrolysis were estimated by two differ-:ent methods: firstly, by oxidation to formic acid and titration and, secondly, through a colorimetric reaction with benzidine. The proportions of sugars found were:

	periodate method	benzidine method
anhydro-rhamnose	23%	26%
anhydro-galactose	27%	24%

These results are in fairly good agreement, and the proportions indicated by the periodate method were further substantiated by a color-:imetric estimation of methylpentose which did not require previous hydrolysis of the polysaccharide. Under the conditions of the experiment, rhamnose reacts with cysteine and sulphuric acid to form a product which can be estimated quantitatively from the absorbance of the solution at 3960A.

A correction is made by subtraction of a reading taken at 4300A. This allows for the presence of hexoses and hexuronic acids which exhibit an absorbance at 4300A equal to that at 3960A, whilst methylpentoses do not contribute at all at a wave length of 4300A.

By this method it was established that the proportion of anhydro-rhamnose was 24%.

Partial Hydrolysis of the Polysaccharide: Isolation and Characterisation of the Neutral Sugars and of the Uronic Acid.

In order completely to establish the identity of the constituent sugars and uronic acid the syrup obtained after partial hydrolysis of the polysaccharide with N-sulphuric acid for seven hours at 100° was investigated. In one experiment it was separated into two fractions, a "neutral sugars" fraction (K) (page 76) and a "barium uronates" fraction (L) (page 76). These were not completely pure, each containing some of the other.

Separation of the "neutral sugars" fraction by partition chromatography on a column of powdered cellulose using n-butanol:water as eluant showed it to contain:

Fraction giving positive Selivanoff test ...	0.8%
L-Rhamnose	9.6%
L-Rhamnose + pentoses	1.7%
D-Galactose	35.1%
Incompletely hydrolysed barium uronates ...	52.8%

L-Rhamnose and D-galactose were isolated in the crystalline state and identified by their melting points, mixed melting points with authentic samples and by their optical rotations. In addition, a characteristic crystalline derivative was prepared for each sugar and was found to have

the correct melting point and mixed melting point.

Attempted fractionation of the residual barium uronates remaining on the top of the column by elution with n-butanol:acetic acid:water, followed by water washing of the column was unsuccessful and these were added to the "barium "uronates" fraction (L).

These combined uronates were converted to the free uronic acids (M) (page 79) with ion-:exchange resin and a portion bydrolysed with 2N-sulphuric acid for 18 hours at 100°. Further quantities of galactose and rhamnose were liberat-:ed (paper chromatography). A second portion of the uronic acids (M) was converted to the methyl ester methylglycoside and the resulting syrup reduced with sodium borohydride. The reduced product was hydrolysed and examined chromatographically. Spots corresponding to galactose, rhamnose and glucose were obtained, and the latter was eliminated on treatment of the reduced, hydrolysed syrup with the specific enzyme glucose oxidase. As glucose was not found in the hydrolysate of the gum, even after vigorous conditions of hydrolysis, before treat-:ment with sodium borohydride, it must have been formed by reduction of glucuronic acid residues.

In a second experiment, after partial hydrolysis, the total hydrolysate was freed from barium with Amberlite IR-120 resin and the uronic acids were converted to the methyl ester, methyl glycosides (and the free sugars to the methyl glycosides) and the mixture reduced in aqueous solution with potassium borohydride. The product was hydrolysed and the neutral sugars (P) (page 82) separated by partition chromatography on a paper column using n-butanol:water as eluant.

Three main fractions and four overlap fractions were collected. Of the pure fractions:

the first was L-rhamnose, characterised as the crystalline 2:5-dichlorophenylhydrazone of correct melting point and mixed melting point.

the second was D-glucose, characterised as the crystalline solid of correct melting point, mixed melting point and optical rotation.

the third was D-galactose, characterised as the crystalline 2:5-dichlorophenylhydrazone of correct melting point and mixed melting point.

The presence of D-glucose in the reduced hydrolysed syrup established the presence of D-glucuronic acid in the polysaccharide.

The quantity of galactose isolated was

relatively greater than that estimated in the original polysaccharide. Although the exact composition of the hydrolysate was uncertain because of difficulties in purification of the fractions and the extent of overlapping, the approximate proportions present were:

L-rhamnose	28%
D-glucose	23%
D-galactose	49%

Quantitative chromatographic methods of estimating sugars which require broad papers, for example by elution and periodate oxidation, are not entirely reliable for estimating glucose in the presence of galactose because of the difficulty of obtaining an adequate separation of these sugars without the possibility of distorting the sugar "band".

For the purpose, therefore, of obtaining a more accurate determination of the relative proportions of the sugars present in (P), an enzymic method was developed in which the glucose was selectively oxidized to gluconic acid, whilst the rhamnose and galactose were unaffected. By measuring the reducing power of the sugars before and after the enzymic oxidation the proportion of glucose present was determined.

The proportion of rhamnose in the mixed

sugars was determined by a conventional quantitat-:ive chromatographic method.

From the results it was deduced that the constituent sugars were present in the following proportions:

glucose	17.7%
rhamnose	24.2%
galactose	58.1%

The low yield of rhamnose and glucose relative to galactose can be explained by the alkalinity of the aqueous sodium borohydride solution used in the reduction. This would be expected to cause partial hydrolysis of the uronic acid esters present; the free uronic acids would then be lost by adsorption on the resins used in the purific-:ation of the product. Since a relatively high proportion of galactose is set free during the partial hydrolysis, loss of product would be expected to affect mainly glucuronic acid and rhamnose-glucuronic acid oligosaccharides. However, additional galactose might have arisen by the reduction of D-galacturonic acid residues in the polysaccharide and although these would also have been partially adsorbed by the resin, some would have been reduced to D-galactose and persisted in the final syrup (P), thus increasing

the proportion of galactose. In view of this and of the occurrence of D-galacturonic acid in other species of Sterculia gum exudates examined, and also because of the difficulty of chromato-:graphically identifying galacturonic acid in the presence of glucuronic acid, it was considered desirable to investigate this possibility further.

The polysaccharide was submitted to a drastic hydrolysis with 2N-sulphuric acid at 100° for 24 hours.

After neutralisation with barium carbonate, the barium uronates were isolated by ethanol precipitation and freed from neutral sugars by repeated dissolution and reprecipitation.

After removal of barium with ion-exchange resin, the uronic acids (21.5% of the poly-:saccharide content of the starting material) in a portion of the resulting syrup were separated into two fractions by partition chromatography on sheets of thick paper, using an acidic eluant.

Two fractions were obtained, the first being identified as D-glucurone by the isolation of its characteristic crystalline derivative with p-nitroaniline with correct melting point and

mixed melting point.

The second fraction, which had the optical rotation of D-glucuronic acid, gave no indication of galacturonic acid in a sensitive colour test with basic lead acetate, and was not oxidized to mucic acid on treatment with bromine water, nitric acid being avoided as oxidant because it would oxidize the trace of contaminating galactose which might have persisted in this fraction.

These results prove that at least the syrup obtained after degradative hydrolysis of the polysaccharide is free from galacturonic acid.

Methylation Studies

The polysaccharide was methylated by three treatments with dimethyl sulphate and alkali, followed by partial removal of inorganic material by dialysis, evaporation and three further methylations of the product. The partly-methylated polysaccharide was converted to the "free-acid" form by acid dialysis, (yield 64%; ash 5.03%; methoxyl 28.4%). The low yield may have been due to mechanical losses caused by faulty construction of the cellophane bags used for dialysis - no evidence of degradation of the polysaccharide was observed.

After removal of the remaining cations with

ion-exchange resin, the partly methylated polysaccharide was converted to the silver salt and esterified by treatment with silver oxide and methyl iodide. Three further treatments with these reagents gave the methylated material (Yield on starting material as polysaccharide 44%; $[\alpha]_D^{18}$ + 68.4°; OMe 42.3%). Further treatment with Purdie reagents failed to raise the methoxyl content. Attempted methylation in dimethyl formamide was unsuccessful.(131) Like the parent polysaccharide, the methylated material was extremely resistant to hydrolysis.

Partial methanolysis of the methylated polysaccharide was achieved by heating with 8% methanolic hydrogen chloride for 18 hours at 100°(94). After hydrolysis of the glycosides with aqueous hydrochloric acid, the solution was neutralised and the product eventually obtained as a mixture of neutral methylated sugars and barium uronates from which the former were extracted with ether.

After a further hydrolysis of the barium uronates with 2N-sulphuric acid for 21 hours at 100°, evidence of degradation was apparent, the product being recovered in diminished yield and containing only a small quantity of ether-soluble

material.

The neutral methylated sugars (29% of the methylated polysaccharide) were separated by partition chromatography on a column of powdered cellulose using n-butanol:light petroleum:water as the eluant. By this means the following fractions were obtained:

Fraction T_1

2:3:4:6-Tetra-O-methyl-D-galactose, which was characterised as the crystalline aniline derivative.

Fraction T_2

A mixture of 2:3:4:6-tetra-O-methyl-D-galactose and 3:4-di-O-methyl-L-rhamnose which was separated by partition chromatography on a column of acid-washed "Celite" using benzene:ethanol:water as eluant. The first component was identified by its analytical constants and the second was isolated in crystalline form and was characterised by its analytical constants and behaviour on electroionophoresis in borate buffer of pH 10.

Fraction T_3

2:3:6-Tri-O-methyl-D-galactose, which was characterised by oxidation to the crystalline lactone with bromine water.

Fraction T_4

3-O-methyl-L-rhamnose, which was obtained in crystalline form and identified by its melting point alone and with an authentic specimen, and by its X-ray powder photograph which was identic-:al with that of the authentic material. In addition, oxidation with bromine water produced a syrupy lactone which had a characteristic optical rotation agreeing with that previously recorded.

The neutral methylated sugars were obtained in the following proportions:

	Proportion of methylated polysaccharide.
2:3:4:6-Tetra-O-methyl-D-galactose	5.7%
3:4-Di-O-methyl-L-rhamnose	0.6%
2:3:6-Tri-O-methyl-D-galactose	7.2%
3-O-Methyl-L-rhamnose	2.3%
Barium uronates also present	4.0%

These proportions correspond to the weight of the fully purified sugars obtained; the original weights from the column were much higher.

The methylated acidic fraction was exceed-:ingly difficult to hydrolyse. Even after methanolysis with 8% methanolic hydrogen chloride for 18 hours at 100° followed by N-hydrochloric acid for 4 hours at 100° and 2N-sulphuric acid

for 21 hours at 100° the resulting syrup was not completely hydrolysed into its constituent sugars and uronic acid. It was, however, separated into three fractions on a column of acid-washed "Celite" using n-butanol:n-butyl acetate:acetic acid:water as eluant.

The first fraction contained methylated uronic acid and neutral methylated monosaccharide. After treatment with methanolic hydrogen chloride the mixed methyl ester, methyl glycosides were reduced with lithium aluminium hydride. After hydrolysis of the reduced glycosides, the result-:ing neutral sugars were partitioned on a column of powdered cellulose using methylethyl ketone:water:ammonia as eluant.

3:4:Di-O-methyl-L-rhamnose was isolated and identified by its analytical constants, electro-:ionophoretic mobility and oxidation with potassium periodate.

2:3:4-Tri-O-methyl-D-glucose was isolated and characterised by its analytical constants and by preparation of the crystalline aniline derivative. As this sugar was not present among the neutral sugars isolated on direct hydrolysis of the methylated gum, it must have

arisen by the reduction of 2:3:4-tri-O-methyl-:D-glucuronic acid.

The second fraction (V_2) was also contamin-:ated with neutral sugars, which were removed by separation on thick paper. The third fraction (V_3) was free from neutral sugars.

Both these fractions were investigated by conversion to the methyl ester, methyl glycosides, reduction in tetrahydrofuran and hydrolysis of the products.

The mixtures of monosaccharides obtained were examined by paper chromatography; each fraction was found to have given a complex mixture of monosaccharides, some of which were identifiable as compounds already characterised, whilst others not previously observed were also found; the results are set out overleaf:

Compo-:nent	Colour of Spot	R_G	Possible Identity	Second Fraction	Third Fraction
(a)	Red-brown	0.85	2:3:4-Tri-O-methyl-D-glucose	Present	Present
(b)	Red-brown	0.71	2:3:6-Tri-O-methyl-D-galactose	Absent	Present
(c)	Lemon-brown	0.56	3-O-Methyl-L-rhamnose	Present	Present
(d)	Red-brown	0.46	Di-O-Methyl hexose	Absent	Present
(e)	Brown	0.28	Rhamnose	Present	Present
(f)	Red-brown	0.23	Mono-O-methyl hexose	Present	Present

The fractions from the methylated uronic acids separation were obtained in the following proportions:

	Proportion of methylated polysaccharide.
First fraction (V_1)	16.6%
Second fraction (V_2)	4.0%
Third fraction (V_3)	1.7%

Reduction of the whole methylated poly-:saccharide with lithium aluminium hydride in tetrahydrofuran followed by remethylation with methyl iodide and silver oxide gave the methylated,

reduced, remethylated polysaccharide which was treated with methanolic hydrogen chloride, then hydrolysed with N-hydrochloric acid. As well as exhibiting the components expected, the hydro-:lysate showed components (d), (e) and (f) (page 50), indicating that these were not the result of the severe hydrolytic conditions employed on the methylated polysaccharide.

Assuming that the sugars were present in the pyranose form in the original polysaccharide (and this assumption is in agreement with the stability of the polysaccharide to acid hydrolysis and to autohydrolysis), then the methylated sugars which have been fully identified indicate that the following structural units are present in the polysaccharide from Brachychiton diversifolium (Sterculia caudata).

(i)	(ii)	(iii)	(iv)
←1.D-Ga	←1.D-Ga.4-	←1.L-Rh.2-	←1.L-Rh.4-
			2 ↓
		(v)	
		←1.D-Glu	

Key: D-Ga:- D-galactose
L-Rh:- L-rhamnose
D-Glu:- D-glucuronic acid.

Galactose residues (i) and (ii).

Both of these have been found in Khaya grandifolia gum (30) and although it also contain-:ed a small quantity of a triply-linked galactose residue this was not considered to be an integral part of the structure, and has been claimed to arise possibly from under-methylation or de-:methylation.

Sterculia setigera gum (41), though similar to the above, contained sufficient triply-linked galactose for the linkage points to be identified as positions 1, 3 and 4; Cochlospermum gossypium gum (29) was similar.

In contrast with these results, galactose linked 1, 3 and 6 is the predominating residue for the other gums examined.

It is, in fact, the only one found in cholla (22) and mesquite (129) gums, whilst gum arabic (128) contains in addition end-group galactose. Cherry (130) and egg plum (124) gums have 1, 3, 6- and 1, 3-linked galactose, whilst damson gum (127) contains in addition 1, 3, 4, 6- and possibly 1, 2, 3, 6-linked galactose.

Rhamnose residues (iii) and (iv).

Both of these may be common to Sterculia

setigera gum, but whilst (iii) was characterised, the branch-point was not completely defined being described as 1, 2, 4- or 1, 3, 4-linked rhamnose or possibly both. Both are, however, common to Cochlospermum gossypium gum, but this had in addition rhamnose end group. Residue (iv) only was found in Khaya grandifolia gum. By contrast with these, gum arabic contained rhamnose attached only on the perimeter of the molecule.

Glucuronic acid residue (v).

The nature of the uronic acid in Brachychiton diversifolium is in contrast with the resemblances already drawn, since the three gums with which it has been compared above, namely, Sterculia setigera, Cochlospermum gossypium and Khaya grandifolia, contain D-galacturonic acid, although the last of these contains in addition 4-O-methyl-D-glucuronic acid, and that as end-:group. Residue (v) has been found in mesquite gum, however, and is common to gum arabic as well as cherry and damson gums, though these three contain also 1,4-linked glucuronic acid. Egg plum and sapote gums contain glucuronic acid linked 1,4- and 1,2- respectively.

The Uronic Acids from the Partly-hydrolysed Polysaccharide.

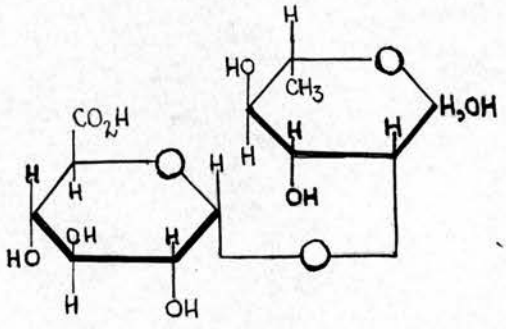
Partial separation of the uronic acids (M, page 79) was achieved by stepwise elution from a column of anion exchange resin with increasing concentrations of acetic acid. Two pure fractions (M_1) and (M_2) were isolated and identified and four other acidic fractions were isolated which were mixtures and which gave glucuronic acid, rhamnose and galactose on further hydrolysis.

Of the two pure fractions (M_1) was identified as glucuronic acid and the other (M_2) had $[\alpha]_D$ + 63° and gave rhamnose and glucuronic acid on hydrolysis and had the correct equivalent weight for an aldobiuronic acid containing these two sugar residues. Partial hydrolysis of a portion with N-sulphuric acid at 100° for 4 hours gave a syrup which on chromatographic analysis showed spots corresponding to rhamnose, glucuronic acid and unchanged M_2. Reduction and hydrolysis of a further portion of M_2 followed by chromatographic analysis of the resulting syrup showed only glucuronic acid and the complete disappearance of rhamnose, indicating that rhamnose carried the free reducing group in the aldobiuronic acid. The remainder of M_2 was methylated and the product

was reduced, with lithium aluminium hydride, to the methylated disaccharide. The resulting syrup was remethylated and hydrolysed. Chromato-:graphic analysis of the product showed two main spots and a slower fainter spot. Separation on thick paper gave syrups corresponding chromato-:graphically and ionophoretically to 2:3:4:6-tetra-:O-methyl-glucose and to 3:4-di-O-methyl-rhamnose and giving rise to glucose and rhamnose respect-:ively on demethylation. A trace of a tri-O-:methyl-glucose which was isolated was considered to arise from undermethylation of the aldobiuronic acid.

These facts point to 2-O-α-D-glucuronosyl-L-rhamnose (I) as the structure of the aldo-:biuronic acid.

(I)



(α-linkage indicated by positive specific rotation.)

That this is correct is supported by the isolation of 3:4:di-O-methyl-L-rhamnose from the hydrolysed methylated polysaccharide and of 2:3:4-tri-O-:methyl-D-glucose from the reduced methylated uronic acids. Additional support is found in chromatographic detection of 2:3:4:6-tetra-O-:methyl-glucose after hydrolysis of the methylated reduced and remethylated polysaccharide. Although 2-O-D-galacturonosyl-L-rhamnose has been isolated from other plant gums (29, 30, 40) and from okra mucilage (12), this is the first reported isolation of glucuronic acid glycosidically linked to the 2-position of L-rhamnose.

Some of the main structural features of the gum exudate of Brachychiton diversifolium have now been determined. From the results of methylation studies, it is clear that end groups of D-galactose and D-glucuronic acid are present while L-rhamnose residues provide the main branch-:ing points in the molecule. The isolation of 2:3:6-tri-O-methyl-D-galactose indicates the presence of 1:4-linked galactose residues in the polysaccharide.

Although D-glucuronic acid is certainly the main acid constituent of the polysaccharide and

no evidence for the presence of galacturonic acid residues has been obtained, the presence of this sugar cannot be completely ruled out in view of the high resistance of the gum and methylated gum to hydrolysis and the consequent degradation when drastic conditions of hydrolysis are employed.

All attempts to separate the polysaccharide into more than a single entity were unsuccessful, but until more evidence is available it is impossible to decide whether or not it consists of a single molecular species.

EXPERIMENTAL

EXPERIMENTAL

General Methods used throughout the Work.

Unless otherwise stated:

Evaporations were carried out at 40° under reduced pressure. Samples for analysis were dried over-night in vacuo over phosphorus pentoxide.

Methoxyl determinations were carried out by a volumetric Zeisel method.

Small scale hydrolyses were carried out by treating samples of the material (ca. 30 mg.) in sealed tubes with sulphuric acid of the stipulated strength at 100°. The hydrolysates were cooled, and neutralised with barium carbonate. After filtration, the solutions were evaporated to dryness.

Paper chromatograms were run with control sugars on Whatman No. 1 paper at a constant temperature of 20°. The solvents used were:

I. Benzene:n-butanol: pyridine:water (1:5:3:3) upper phase.

II. Ethyl acetate:pyridine:water (10:4:3)

III. n-Butanol:ethanol:water (4:1:5) upper phase.

IV. Ethyl acetate:acetic acid:formic acid:water (18:3:1:4)

V. n-Butanol:acetic acid:water (4:1:5) upper phase.

VI. Benzene:ethanol:water (169:47:15) upper phase.

Papers were sprayed with a saturated aqueous

solution of aniline oxalate and the spots were developed at 120°. p-Anisidine hydrochloride (3%) in n-butanol containing a little stannous chloride was used under similar conditions.

Rates of movement of sugars on the chromatograms are described in terms of the following ratios:

R_F = Distance travelled by sugar / " " " solvent front

R_G = Distance travelled by sugar / " " " 2:3:4:6-tetra-O-methyl-D-glucose

R_{Gal} = Distance travelled by sugar / " " " D-galacturonic acid

Electroionophoresis experiments were carried out in an apparatus modelled on that of Foster (85). Whatman No. 1 paper was used in a sodium hydroxide-boric acid buffer of pH 10. Time for equilibrat-:ion was 15 minutes. After running for 4-6 hours (500 V., 12.5 mA) the paper was dried, and the sugars located by the aniline oxalate spray (+5% acetic acid).

Demethylations were carried out on partly-:methylated derivatives by treatment with hydro-:bromic acid in a sealed tube at 100° for 5-10 minutes, followed by neutralisation with silver carbonate and deionisation of the filtrate either by evaporation and ethanol extraction or by

passage through a column of cation exchange resin (Amberlite IR-120, H). The products were identified chromatographically.

Tetrahydrofuran for reductions with lithium aluminium hydride was dried by allowing it to stand over sodium for 1-2 days, then refluxing and distilling from sodium. The distillate was then refluxed with lithium aluminium hydride for 30 minutes and distilled, moisture being excluded at all stages by fitting calcium chloride tubes. A saturated solution of lithium aluminium hydride in tetrahydrofuran (ca. 0.12g. per ml) was prepared by refluxing the two for 5-10 minutes and removing excess of the solid on the centrifuge in stoppered tubes.

Thick paper sheets (Whatman 3MM) for chromatographic separation of small amounts of sugars were subjected to a preliminary extraction with hot benzene:ethanol (1:1) in a Soxhlet apparatus.

Brachychiton diversifolium: the Crude Gum.

The gum, obtained as translucent nodules with a slight brown colouration, was contaminated with bark and had a resinous odour. In contact with water it swelled forming a thick gelatinous mucilage.

Three nodules of differing appearance were powdered separately and dried to constant weight over phosphorus pentoxide at 60° under reduced pressure (loss of weight: 17%). Analysis showed that the samples were not significantly different in composition, and the following results are averages of the three separate investigations.

Ash content

A weighed sample was completely incinerated in a tared platinum crucible, which was then re-:weighed.

Found: Ash = 4.1%.

Methoxyl

Found: Methoxyl = 1.4%.

Acetyl

A weighed sample was submitted to alkaline hydrolysis according to Clark (57). After acidification, the liberated acetic acid was

steam-distilled and titrated with standard sodium hydroxide.

Found: Acetyl = 19.3% (as OAc)

Reducing power

The gum did not reduce Fehling's solution on heating. No insoluble complex was formed.

Elements present

A Lassaigne sodium test showed the absence of nitrogen, halogens and sulphur. Absence of nitrogen was confirmed by a Kjeldahl estimation, the sample giving a result indistinguishable from that of the "blank".

Complete Hydrolysis and Chromatography of the Products.

A sample of gum was heated for 24 hours at 100° with 2N-sulphuric acid. The solution, which had become dark brown, was neutralised with barium carbonate and the insoluble barium salts removed by filtration. The filtrate was treated with decolourising charcoal, then evaporated to a syrup which was examined by paper chromatography (solvent I.). Investigation with a variety of spray reagents revealed spots corresponding in position and colour to glucurone, rhamnose, galactose and barium uronates; the latter remain-

:ing at the starting line. Faint spots corres-:ponding to xylose and arabinose were also detect-:ed.

After removal of barium from the hydrolysate with ion-exchange resin (Amberlite IR-120.H) chromatographic examination in an acidic solvent (solvent IV.) gave no evidence of acidic oligo-:saccharides.

Solubility in Water and Precipitation of Separate Fractions

The gum (10g.) was stirred with water (500 ml.) at 50° for 30 minutes. Bark was removed by squeezing through muslin and the pro-:duct submitted to centrifugation. The super-:natant liquors (200 ml.) were acidified to pH2 by addition of dilute hydrochloric acid, and ethanol (4 volumes) was added. After standing at 0° for 18 hours the solution had deposited a white gelatinous precipitate which was removed on the centrifuge, then dissolved in water. After reprecipitation in acetone and dissolution in water (three times), the solution was free from chloride ions and the product was then isolated by freeze-drying as a white fibrous solid (0.075 g.) $[\alpha]_D^{18}$ + 55° (c. 0.18 in N-sodium

hydroxide). Further extraction of the original mucilage with water (300 ml.) gave a second, (0.05g., reprecipitated with 4.5 volumes of acetone), and a third fraction, (0.01g., reprecip-:itated with 10 volumes of acetone). These were isolated as above.

Complete hydrolysis and chromatography showed that the three fractions were not signific-:antly different from the crude gum in composition.

Dissolution of the Gum and Fractional Precipit-:ation of the Polysaccharide

The crude gum (4.0g.) was shaken for 18 hours with N-sodium hydroxide solution (200 ml.) in an atmosphere of nitrogen. After acidific-:ation (pH 2) with hydrochloric acid, ethanol was added gradually. By this means the poly-:saccharide was precipitated in discrete fract-:ions which were isolated, triturated free from chloride ion with ethanol, then washed with ether and dried over phosphorus pentoxide under vacuum. The fractions isolated were as follows:

(A) 1.41 g. (1.5 volumes of ethanol added)
(B) 0.15 g. (2.0 volumes " " ")
(C) 0.03 g. (5.0 volumes " " ")
(D) 0.01 g. (7.0 volumes " " ")

Further addition of ethanol resulted in precipitation of sodium chloride. Each fraction was examined by complete hydrolysis and chromato-:graphy, when (A) and (B) were found to be identical in containing substances corresponding to glucurone, rhamnose, galactose and barium uronates, whilst (C) and (D) were similar, but showed in addition traces of pentoses correspond-:ing to xylose and arabinose.

Brachychiton diversifolium Gum: the Isolation of the Pure Polysaccharide

The crude gum, finely powdered in the ball mill, was then purified in batches according to the following procedure:

The gum (4.0 g.) was shaken for 18 hours with N-sodium hydroxide solution (200 ml.) in an atmosphere of nitrogen. The solution was chilled and acidified (pH 2) by addition of hydrochloric acid (50%). Fragments of bark were removed on the centrifuge and the polysaccharide was then precipitated by slow addition of the solution to ethanol (3 volumes) with vigorous stirring.

The precipitate was removed by filtration, then redissolved in water ("Ato-mix" disperser), acidified and precipitated in ethanol as before.

After isolation, the polysaccharide was triturated with ethanol until free from chloride ions, washed with ether, then dried to constant weight over phosphorus pentoxide (60°/12 mm pressure): yield 2.2 g.

Properties of the Pure Polysaccharide

The polysaccharide, isolated as a white fibrous solid had $[\alpha]_D^{17}$ + 69° (c. 0.40 in N-:sodium hydroxide); ash: 0.83%; acetyl: nil.

Methoxyl content

A sample was freed from adhering ethanol by dissolution in water and reprecipitation by addition to glacial acetic acid. After removal on the centrifuge, the polysaccharide was re-:dissolved in water and freeze-dried.

Found: Methoxyl, nil.

Electrophoresis

The polysaccharide (0.05 g.) in borate buffer of pH 10 (10 ml.) gave a single symmetrical peak when examined in the Antweiler micro-:electrophoresis apparatus (1.5 mA, 35 V., 10 mins.)

Equivalent weight

A weighed quantity of polysaccharide was allowed to stand with excess of standard sodium hydroxide solution until it had dissolved. The

excess of alkali was then determined by titration with standard acid in presence of phenolphthalein and corrected for a "blank" determination run concurrently.

Found: Equivalent weight = 342.

Uronic anhydride (60)

A weighed amount of polysaccharide was quantitatively decarboxylated by heating with 19% hydrochloric acid, and the carbon dioxide liberated was determined by absorption in standard alkali, the excess of alkali being determined by titration, after addition of barium chloride solution.

Found: Uronic anhydride = 50.1% (average of two determinations).

Autohydrolysis of the Polysaccharide

The polysaccharide (1.46 g.) was heated with water (150 ml.) at 95-100° under reflux until it dissolved (about 1 hour). The course of heating after this time was followed by chromato-:graphy of aliquot portions of solution and by hypoiodite oxidation (99): the cooled solution (10.00 ml.) was added to a buffer solution (sodium hydroxide: disodium hydrogen phosphate) of pH 11.4 (25.0 ml.) followed by addition of 0.1 N-iodine (5.00 ml.). The tube was stoppered

and after two hours sulphuric acid (3%: 50 ml.) was added and the liberated iodine titrated with standard sodium thiosulphate solution. Turbidity of the solution prevented polarimetric readings.

Time of heating	Iodine No. (mls. 0.1 N-iodine taken upper g. of polysaccharide.)
2 hours	0.2
4 "	0.3
8 "	1.0
12 "	2.7
24 "	8.1

No monosaccharides were detected in the aliquots (paper chromatography). After 24 hours the solution was cooled and the polysaccharide precipitated by pouring into ethanol. The ethanolic solution after concentration was found to contain traces of galactose, rhamnose and pentoses (paper chromatography).

The Course of Acid Hydrolysis of the Polysaccharide.

The polysaccharide (2.00 g.) was heated with N-sulphuric acid (100 ml.) at 100°. At hourly intervals samples were tested for iodine uptake (2.00 ml. aliquot). Optical rotations were taken after two hours' heating and from then onwards due to the initial turbidity of the solut-:ion.

Time (hours)	1	2	3	4	5	6	7	8	9	13
$[\alpha]_D^{16}$	-	+104°	+102°	+99°	+98°	+91°	+90°	+90°	+86°	+77°
Iodine No.	29	40	46	51	52	51	50	51	54	59

The iodine uptake was determined by the method used in the autohydrolysis experiment above, except that to avoid changing the pH of the buffer solution, the sample of test solution was first neutralised by the addition of a calculated amount of standard alkali.

Complete Hydrolysis of the Polysaccharide and Quantitative Chromatography of the Neutral Sugars

A known weight of polysaccharide was hydrolysed at 100° with 2N-sulphuric acid for 24 hours. After cooling, a weighed amount of ribose was added (for first method of estimation only). The mixture was neutralised with barium carbonate and the insoluble barium salts removed by filtrat-:ion and washed. After treatment with decolour-:ising charcoal, the solution was again filtered. The filtrate and washings were evaporated to dryness, taken up in water and again filtered and evaporated to yield a residue which was dissolved in a known volume of water. An aliquot portion of the sugar solution was accurately delivered on to the chromatography paper (Whatman No. 1) with an Agla semi-micro syringe, and the paper irrigated with solvent No. I. It was then dried at room temperature for 24 hours in vacuo

and the sugars located by spraying side and central strips with aqueous aniline oxalate.

Sections of the chromatogram corresponding to rhamnose, ribose and galactose were cut out along with a "blank" strip, and the sugars were estimated as follows:

(i) Estimation by periodate oxidation to Formic Acid (74).

The paper strips were extracted under reflux with water (5 ml.) for 45 minutes. The solutions were heated with 0.25 M sodium metaperiodate solution for 20 minutes at 100° in stoppered tubes (8" x 1"), the upper parts of which were cooled by condenser coils. Ethylene glycol (neutral; 0.3 ml.) was added to the cooled solutions and after five minutes the formic acid present was estimated with 0.01 N carbonate-free sodium hydroxide solution using methyl-red indicator, the values being corrected for the "paper blank".

Found: the polysaccharide contained 23% anhydro-rhamnose and 27% anhydro-galactose (average result of four determinations).

In a typical experiment utilising the polysaccharide (0.178 g.) and ribose (0.0236 g.) the hydrolysate was made up to 10 ml. with water

and an accurately-measured volume (0.200 ml.) applied to the chromatogram. The results obtained were:

Sugar	0.01N Sodium Hydroxide titre (ml.) (blank 0.040 ml.)	Estimated Wt. of Sugar in 10 ml. of Solution (g.)	Wt. of anhydro-sugar in 10 ml. of solution (g.)	%age of anhydro-sugars in the poly-:saccharide*
Rhamnose	1.270	0.025	0.022	23.5
Galactose	1.596	0.028	0.025	26.5
Ribose	1.160	0.021	-	-

* (Allowing 50% of starting weight as uronic anhydride).

Proportion of pentoses present: pentoses were not detected on the guide strips and sections of the chromatogram which might have contained pent-:oses were found not to differ significantly from the "paper blank" when examined in the above manner.

Recovery from Hydrolysis:

Assuming that each sugar is lost by absorption on the barium salts to the same extent as the ribose; the corrected weights of sugars in 10 ml. of solution are:

anhydro-rhamnose	0.025 g.
anhydro-galactose	0.028 g.
Total	0.053 g.

Since the uronic anhydride constitutes 50.1% of the polysaccharide, the total weight accounted for is:

Neutral sugars	0.053 g.
Uronic anhydride	0.089 g.
Total	0.142 g.

This represents a recovery of 80% of the weight of the polysaccharide hydrolysed.

(ii) Colorimetric Estimation with benzidine and glacial acetic acid (98).

The paper strips were extracted under reflux with 90% methanol and the solutions were evaporat-:ed to dryness under reduced pressure. The syrups obtained were dissolved in a known volume of water and portions (1.00 ml.; containing not more than 600 µg. of sugar) were placed in test-:tubes (7" x 1") provided with small glass spheres to prevent entry or loss of water. The benzidine reagent (5.00 ml. of a 0.2% solution of benzidine in glacial acetic acid containing 0.1% stannous chloride) was added from a burette. After mixing, the tubes were heated in a vigorously-boiling water bath for 30 minutes, then cooled in running water. The colour was then measured in a Hilger "Spekker" photoelectric absorptio-:meter using a violet filter (Ilford No. 601,

wavelength 425 mμ) and a 0.5 cm. glass cell. The weight of sugar in the test solution was obtained by reference to a calibration curve prepared from standard solutions run concurrently.

Found: the polysaccharide contained 26% anhydro-rhamnose and 24% anhydro-galactose (average of two determinations).

In a typical experiment utilising the polysaccharide (0.0988 g.), the hydrolysate was made up to 5.00 ml. with water and an accurately-:measured volume (0.250 ml.) applied to the chromatogram.

(a) Readings of standard solutions

Galactose (μg.)	Spekker reading	Rhamnose (μg.)	Spekker reading
250	0.295	130	0.155
280	0.305	287	0.340
501	0.580	443	0.660

(b) Readings of test solutions

Galactose: 0.450 corresponding to 347 μg. of anhydro-sugar

Rhamnose: 0.600 " " 379 μg. of anhydro-sugar

Thus the proportions of anhydro-sugars present are:-

Galactose:Rhamnose = 1:1·09

Allowing for 50.1% uronic anhydride in the poly-

:saccharide, the proportions of neutral sugars present are:

anhydro-galactose 23.9%
anhydro-rhamnose 26.0%

The Colorimetric Estimation of Rhamnose in the Polysaccharide (96)

To an aqueous solution of the poly-:saccharide (1.00 ml.) containing 2-15 μg. of rhamnose, there was added with cooling in ice a mixture (4.50 ml.) of water (1 volume) and con-:centrated sulphuric acid (6 volumes). An aqueous solution of cysteine hydrochloride (3%: 0.10 ml.) was then added with shaking. (The solution of cysteine hydrochloride was stored in a brown bottle at 0° and used within 48 hours after time of preparation.) The extinction coefficients of the solution at 3960A and 4300A were determined against a water blank (Unicam spectrophotometer S.P. 500) and the difference ($\Delta E_{3960-4300}$) calculated. Since the figure so obtained was directly related to the weight of rhamnose present in the original solution, this latter quantity could be calculat-:ed by reference to the difference in extinction coefficients of a standard rhamnose solution run

concurrently.

Found: anhydro-rhamnose content of the polysaccharide = 23.9% (average result).

In a typical estimation employing the solutions:

(F) 49.8 μg. of polysaccharide per ml.

(G) 7.21 μg. of rhamnose per ml.

the following results were obtained:

	(F)	(G)
E_{3960}	0.310	0.170
E_{4300}	0.068	0.039
$\Delta E_{3960-4300}$	0.242	0.131

From (G), a difference in extinction coefficients of 0.131 corresponds to a rhamnose content of 7.21 μg. per ml. (or 6.42 μg. anhydro-rhamnose per ml.) The polysaccharide solution (F) thus contains 11.9 μg. of anhydro-rhamnose per ml.

Therefore, anhydro-rhamnose content of the polysaccharide = 23.9%, whilst anhydro-galactose content = 26.0% by difference (allowing for 50.1% uronic anhydride).

Partial Hydrolysis of the Polysaccharide

(a) Separation and Characterisation of the Neutral Sugars.

The polysaccharide (9.45 g.) was hydrolysed

with N-sulphuric acid (500 ml.) for 7 hours at 100°. The solution was cooled then made slightly alkaline with barium hydroxide solution, excess of alkali being removed immediately with carbon dioxide. Insoluble barium salts were removed on the centrifuge and the supernatant liquors and washings were evaporated to dryness under reduced pressure. The residue was extracted with cold water and the extract filtered and poured into well-stirred methanol (10 volumes). The precipitated barium salts were exhaustively extracted with hot methanol and the total methanolic liquors evaporated to a syrup (K) (3.11g.), leaving the barium uronates (L) (5.38g.) as an amorphous solid.

Fractionation of the Neutral Sugars by Partition Chromatography on a Cellulose Column.

A cellulose column (850 x 27 m.m.) was washed with water, n-butanol and finally n-butanol two-thirds saturated with water. The syrup (K) was dissolved in the minimum of water and applied to the top of the column which was developed with ethanol (250 ml.) and subsequently with n-butanol two-thirds saturated with water. The eluant was collected in 7-8 ml. portions by means of an

automatic fraction-cutter. Every tenth tube was evaporated and examined chromatographically. Tubes containing the same sugar were then combined together with their water-washings and evaporated to dryness. Initial purification was effected by cold-water extraction when a wax-like material, possibly degraded cellulose was left behind.

When galactose had been completely eluted the solvent was changed to n-butanol:acetic acid: water (4:1:5, organic phase) in an attempt to fractionate the uronic acids. No fractionation was effected, however, and the residual uronic acids were eluted from the column with water.

Fractions obtained in the Separation of Neutral Sugars

Fraction	Tubes	Colour of Spot	R_F	Weight after purification (g.)
K_1	150-290	Brown	0.78	0.021
K_2	310-390	Yellow	Varies, but identical with rhamnose.	0.260
K_3	391-830	"	"	0.046
		Pink	0.39	
		Pink	0.34	
K_4	1100-1737	Brown	0.24	0.951
K_5	Column washings			1.430
			Total	2.708 g.

Chromatographic solvents I and II were used, R_F values are related to the former.

Examination of the Fractions.

Fraction K_1 Syrup 0.021 g.

This gave a positive Selivanoff test.

Fraction K_2 0.260 g. $[\alpha]_D^{20}$ + 8.4° (c. 1.07 in H_2O)

This fraction crystallised on removal of solvent. Recrystallisation was effected from moist butanol yielding L-rhamnose hydrate m.p. 88-90° (not depressed on admixture with an authentic sample). Treatment with a saturated alcoholic solution of benzoylhydrazine (40) gave a crystalline hydrazone m.p. 186 -189° (decomp.) undepressed on admixture with an authentic specimen.

Fraction K_3 0.046 g.

Three components were observed in this fraction (paper chromatography) corresponding to rhamnose, xylose and arabinose. Visual inspection of the intensities of the spots suggested that rhamnose comprised the bulk of the fraction with xylose and arabinose having a combined weight not exceeding 0.015 g.

Fraction K_4 0.951 g. $[\alpha]_D^{20}$ +83° (c. 3.55 in H_2O).

This fraction crystallised on removal of solvent. Recrystallisation from methanol

yielded D-galactose m.p. 163-165° (not depressed on admixture with an authentic specimen.) The identity of the sugar was further established by the preparation of the diethylmercaptal (101) having m.p. 140-141° (not depressed on admixture with authentic galactose diethylmercaptal).

Fraction K_5 1.430 g.

This fraction, eluted partly with n-butanol: acetic acid:water and finally by washing with water was added to the material (L) (page 76).

(b) Examination of the Barium Uronates.

The barium uronates (L + fraction K_5) (6.81g.) were dissolved in water and converted to the free uronic acids by passage through a column of ion-:exchange resin (Amberlite IR-120, H). Removal of solvent gave an amorphous solid (4.66g.) (M) of equivalent weight 338 and iodine number 45.4). Hydrolysis of a small portion (2N-sulphuric acid at 100° for 18 hours) followed by neutralisation with barium carbonate showed rhamnose and galact-:ose to be present (paper chromatography).

A portion (0.20g.) was boiled with methanolic hydrogen chloride (4%) for 7 hours. After

neutralisation with silver carbonate, filtration and evaporation, a syrup (0.15 g.) was obtained which was reduced by addition, in aqueous solution (2 ml.), to potassium borohydride (0.1g.) in water (1.5 ml.). After 2 hours, excess of acetic acid was added and ions removed on a mixed-bed resin column (Amberlite IR-120,H and IR-4B,OH). The eluant was partly evaporated, made Normal with sulphuric acid, heated for 7 hours at 100° then cooled, and neutralised with barium carbonate. Filtration and evaporation gave a syrup (0.07 g.) containing galactose, rhamnose and glucose, this last being eliminated on treatment with the specific enzyme glucose oxidase (paper chromato-:graphy). Quantitative chromatography by the periodate method (page 70) showed that the ratio of total hexose:rhamnose was 3.3:1 by weight.

The Isolation and Characterisation of Glucose after Reduction of the Partly-:hydrolysed Polysaccharide and the Estimation of the Glucose by Selective Enzymic Oxidation.

A fresh quantity of polysaccharide (8.2g.) was hydrolysed with N-sulphuric acid (300 ml.) for 7 hours at 100°. After cooling, the solution

was neutralised with barium carbonate and filtered. The filtrate was evaporated to dryness, the residues extracted with cold water and the extracts evaporated to a pale-brown amorphous solid (8.2g.), which was then dissolved in water and deionised by ion-exchange resin (Amberlite IR-120,H), yielding a solution of neutral sugars and uronic acids. The solution was evaporated to a syrup which was dried over phosphorus pentoxide under reduced pressure then boiled with methanolic hydrogen chloride (4%) for 6 hours. After cooling, the solution was neutralised with silver carbonate, filtered and the methanol distilled. The residue was dissolved in water (50 ml.) and added slowly and with stirring to a solution of potassium borohydride (4g.) in water (60 ml.) then left for 2 hours.

Excess borohydride was destroyed by addition of dilute acetic acid, and ions were removed by passage through columns of ion-exchange resins (Amberlite IR-120H and IR-4B, OH). The solution was evaporated to dryness, traces of borate being removed by repeated evaporation with methanol. The syrup finally obtained (5.1g.) was hydrolysed by heating with N-sulphuric acid for

7 hours at 100°. After cooling, neutralising with barium carbonate and working-up in the usual way a syrup (P) (4.4g.) composed of neutral sugars was obtained.

(i) Separation of the Constituents

A portion of the syrup (P) (2.19g.) was dissolved in aqueous ethanol (1:1, 20 ml.) and applied to a Grycksbo filter paper column (L.K.B. 3391). After development with n-butanol (200 ml.)n-butanol undersaturated with water was used as eluant (prepared by saturating n-butanol with water at 0° and allowing the separated organic phase to warm to room temperature before use). The solution issuing from the column was collected in 25 ml. fractions at a flow-rate of 80 ml. per hour, examined chromatographically and bulked in the usual manner. The fractions so obtained are listed below. Since, contrary to expectation from the results of pre-washing the column, the fractions were contaminated with a persistent cloudiness, which prevented the recording of optical rotations, only the fraction (P_5) corresponding to glucose was clarified by a laborious process of evaporation, drying out and

dissolution in water or ethanol. The other fractions are reported according to paper chroma-:tographic analysis, the major fractions being characterised as crystalline derivatives. The contaminant may have been degraded polythene since this substance was used to heat-seal the column after the failure of paraffin wax recommended by the manufacturers.

Fractions obtained.

P_1. Tubes 200-414. 0.478g. L-rhamnose.

Characterised as the 2:5-dichlorophenyl-:hydrazone m.p. 171° (not depressed on admixt-:ure with a specimen of the authentic L-rhamnose derivative).

P_2 Tubes 415-521. 0.036g. Rhamnose and xylose.

Visual examination suggests the proport-:ions 4:1 respectively for these sugars.

P_3 Tubes 522-589. 0.020g. Rhamnose, xylose and arabinose in the ratio 4:1:3 (visual estimation).

P_4 Tubes 590-640 0.019 g. Arabinose and glucose in the ratio 1:2 (visual estimation)

P_5 Tubes 641-736. 0.133 g. D-glucose.

On purification of a portion (0.030 g.) and recrystallisation from ethanol and petroleum-

ether crystals were obtained (0.0078g.) having $[\alpha]_D^{18}$ + 53° (c. 0.70 in H_2O) equilibrium value, and m.p. 148-149° (not depressed on admixture with D-glucose which had been recrystallised in a similar manner).

P_6 Tubes 737-751. 0.563g. Glucose and galactose in the ratio 1:1 (visual estimation).

P_7 Tubes 751-951 0.631 g. D-galactose.

Characterised as the 2:5-dichlorophenyl-:hydrazone m.p. 196-197° (not depressed on admixture with a specimen of the authentic D-galactose derivative).

(ii) Quantitative Analysis of Syrup (P)

The Estimation of Glucose in presence of Galactose and Rhamnose

The method is based on the determination of the reducing power of the mixed sugars towards the Somogyi reagent (107) before and after treat-:ment with glucose oxidase (108), an enzyme which catalyzes the oxidation of glucose to the non-:reducing gluconic acid (106). Preliminary experiments established that the enzyme preparat-:ion (commercial "Dee-O" manufactured by the Takamine Corporation) interfered with the inter-:action between the Somogyi reagent and the reducing

sugars. This effect was eliminated by deprotein-:isation of the incubated digests with zinc sulphate and barium hydroxide solutions before the estimation of reducing power. In addition, how-:ever, the enzyme showed slow oxidative activity towards galactose. Whilst this reaction was appreciable after 16-18 hours, it was not signif-:icant after an incubation period of 3 hours which was sufficient for the complete oxidation of glucose under the experimental conditions employed. This was verified by the fact that a prepared sample containing slightly more glucose than the test solution was completely oxidized; in addition paper chromatography of the test solution after incubation showed only rhamnose and galactose.

Experimental Procedure

An aqueous preparation (1.00 ml.) of glucose oxidase (0.075 g. in 10 ml. of water) was added to an aliquot portion (5.00 ml.) of the mixed sugars solution. The mixture was incubated for 3 hours at 36°, then water (0.40 ml.) was added, followed by zinc sulphate solution and barium hydroxide solution (0.30 ml. each) which had been prepared according to Nelson (109). The pre-

:cipitate was removed on the centrifuge and a portion of the supernatant solution (5.00 ml.) withdrawn for the estimation of reducing power by the Somogyi method (107). A "blank" determinat-:ion was carried out simultaneously in exactly the same manner, except that the enzyme preparat-:ion was inactivated by heating at 100° for 10 minutes before use.

Estimations of Glucose

Reducing powers are expressed as ml. 0.01 N-sodium thiosulphate solution and in each instance have been calculated so as to refer to a 5.00 ml. aliquot of the original solution of mixed sugars.

Control solutions:

Q		R	
0.60 mg. rhamnose	per	0.55 mg. rhamnose	per
1.49 mg. galactose	5.00 ml.	1.30 mg. galactose	5.00 ml.
0.25 mg. glucose	aliquot	0.41 mg. glucose	aliquot

Test solution:

Syrup (P) (45.2 mg.) was dissolved in water and made up to 100 ml. giving solution (S) (5.00 ml. is equivalent to 2.26 mg. of syrup (P).

	Q	R	S
Reducing power of "blank"	6.55 ml.	6.41 ml.	5.29 ml.
Reducing power of incubated solution	5.65 ml.	4.90 ml.	3.98 ml.
Decrease in reducing power after incubation	0.90 ml.	1.51 ml.	1.31 ml.
Glucose per 5.00 mls. mixed sugars solution	0.26 mg.	0.44 mg.	0.39 mg.
Recovery of glucose	104%	107%	-

Found: Percentage of glucose in syrup (P) = 17.7% (average of two determinations).

Proportion of Rhamnose in the Syrup

Portions of the syrup (P) were examined by quantitative chromatography. From each chromatogram two strips were cut and eluted, one for rhamnose and one for total hexose (galactose and glucose); a "blank" determination was also carried out. The sugars were estimated by the periodate method.

Found: rhamnose content of the syrup = 24.2%

total hexose = 75.8%

(identical result from two determinations; percentages are determinated from the ratios of the weights of sugars)

Composition of the Syrup

From the above results and neglecting traces of pentoses, the syrup comprises:

D-glucose 17.7% (by enzymic oxidation)
L-rhamnose 24.2% (by quantitative chromatography)
D-galactose 58.1% (by difference).

Found for the syrup: $[\alpha]_D^{18}$ + 55° (c. 1.10 in H_2O)

The above composition requires $[\alpha]_D^{20}$ + 58°.

Separation and Identification of the Uronic Acid produced on Complete Hydrolysis.

The crude gum (12.5 g.; equivalent to 6.85 g. of pure polysaccharide) was hydrolysed completely as before (page 62). The neutralised filtrate was evaporated to dryness and the residues were extracted with cold water giving a dark-brown solution, which was treated with decolourising charcoal and again filtered. The solution (100 ml.) was poured slowly into ethanol (500 ml.) with stirring, and the precipitated barium uronates removed on the centrifuge. These were redissolved in water, reprecipitated in ethanol, and isolated as before. The barium salts were

found to contain a trace of galactose (paper chromatography). The salts were dissolved in water (50 ml.) and freed from barium by passage through a column of ion-exchange resin (IR-120, H). The solution of free uronic acids on evaporation gave a syrup (1.47 g.) of which a portion (0.2 g.) was separated on sheets of thick paper (Whatman 3 MM; solvent IV) yielding two fractions which were eluted with cold water and evaporated to dryness.

Fraction H. 0.039 g.

This material, which was chromatographic-:ally identical with glucurone was converted to the p-nitroaniline derivative by the method of Hamilton, Spriestersbach and Smith (123). Yellow crystals (0.020 g.) were obtained which were tiled; giving after recrystallisation from methanol, yellow needles, m.p. 128-130° (with slight decomposition) not depressed on admixture with the authentic p-nitroaniline derivative of D-glucurone.

Fraction J. 0.116 g. $[\alpha]_D^{18}$ + 33.6° (c. 1.16 in H_2O)

The chromatographic behaviour of this material showed it to be identical with glucuronic acid ($[\alpha]_D$ + 36°) and/or galacturonic acid ($[\alpha]_D$ + 51°).

On treatment of a portion with basic lead acetate solution according to Ehrlich (105) a yellow-:brown precipitate similar to that from glucuronic acid and distinct from the brick-red precipitate given by a "control" sample of galacturonic acid was observed. The remainder of the fraction (ca. 0.10 g.) was tested for galacturonic acid (35) by adding water (5 ml.) and bromine (1 ml.). After standing at 30-35° in a stoppered flask for 4 days, the solution was aerated to remove excess of bromine and evaporated to dryness. The residue was dissolved in N-sodium hydroxide solution, filtered, and acidified by dropwise addition of concentrated hydrochloric acid. No precipitation was observed on prolonged standing.

Methylation of the Polysaccharide

The crude gum (40 g.) (equivalent to 22 g. of pure polysaccharide) was purified by the standard method (page 65), except that the poly-:saccharide after one precipitation in acid ethanol (pH 4) was pressed-out on a Buchner funnel, and methylated as follows:

The polysaccharide was dissolved in sodium hydroxide solution (30%; 600 ml.), in an atmos-

:phere of nitrogen which was maintained during all further methylations involving sodium hydroxide, and the solution cooled. Dimethyl sulphate (270 ml.) was added dropwise over 8 hours with vigorous stirring, the temperature being kept below 15°. After 18 hours, sodium hydroxide solution (30%; 600 ml.) and dimethyl sulphate (270 ml.) were added dropwise over 6 hours. On the following day a solution of sodium hydroxide 180g.) in water (200 ml.) and dimethyl sulphate (270 ml.) were added as before over 8 hours. After chilling, the solution was just acidified by cautious addition of aqueous sulphuric acid (50%), dialysed against running water for 24 hours, and evaporated to 200 ml. bulk. The methylation was repeated three times with sodium hydroxide (30%; 600 ml.) and dimethyl sulphate (270 ml.), then the solution was acidified as before and dialysed for 4 days. The pH was then adjusted to 2 with sulphuric acid and after addition of a further equal volume of acid, the solution was dialysed free from sulphate ions and evaporated to an amorphous solid (14.0g.) having ash content 5.03% methoxyl 28.4%. A portion of the partly-methylated polysaccharide (9.0 g.) was dissolved in water and deionised with

ion-exchange resin (Amberlite IR-120,H) then stirred overnight with an 8-10 fold excess of silver carbonate. After filtration and freeze-drying, the amorphous silver salt was triturated with methyl iodide (50 ml.) then refluxed for 2 hours with the addition of silver oxide (4 g.) in portions (0.5 g.). The product was isolated by filtration and evaporation and after three methylations according to Purdie with methyl iodide and silver oxide yielded the fully-methylated polysaccharide (6.2g.), $[\alpha]_D^{18}$ + 68.4° (c. 1.17 in $CHCl_3$) OMe 42.3%. Further treatment with Purdie's reagent failed to increase the methoxyl content.

The Effect of Dimethylformamide on Methylation with Purdie's Reagents.

The partly-methylated polysaccharide (0.1g.; esterified; OMe 32.8%) was dissolved in anhydrous dimethylformamide (1 ml.) and methyl iodide (2 ml.) and silver oxide (0.1g.) were added. The flask was stoppered and allowed to stand with occasional shaking; two further portions of silver oxide (0.1g. each) were added over 3 days. A further addition of silver oxide was then made and the flask was mechanically shaken for 24 hours. The mixture was filtered, the residues being washed with dimethylformamide,

then with chloroform. Filtrate and washings were combined, allowed to stand overnight at 5°, then filtered. Chloroform was removed by reduced-:pressure distillation at the water pump, and residual dimethylformamide was distilled under high vacuum (bath temp. 40°/18m.m.). The product (0.097 g.) had OMe 33.7%, and was not further examined.

Hydrolysis of the Methylated Polysaccharide; Separation of the Neutral Sugars from the Uronic Acids.

The methylated polysaccharide (6.0g.) was heated for 18 hours with 8% methanolic hydrogen chloride (150 ml.) at 100° in a sealed tube. After neutralisation with silver carbonate, the solution was filtered and evaporated to a syrup which was hydrolysed with aqueous hydrochloric acid (4%) for 6 hours. The solution was neutral-:ised with silver carbonate, filtered and treated with hydrogen sulphide to precipitate silver ions. After a further filtration the solution was made alkaline with barium hydroxide, excess of alkali being removed by passage of carbon dioxide through the solution. The precipitate was removed by filtration and the solution evaporated to a viscous syrup (5.73 g.) from which the neutral methylated sugars were extracted by refluxing

with dry diethyl ether (4 x 100 ml.; 30 mins. each). Evaporation of the ethereal extracts gave a syrup (T) (1.54 g.) The residual barium salts (3.99g.) were subjected to further hydrolysis at 100° with sulphuric acid (2N) for 21 hours. After neutralisation of the cooled solution with barium carbonate and filtration, the residues were extracted exhaustively with water, first at 20° and then at 40° until the extracts gave no positive Molisch test. Evaporation of the extracts gave a brown amorphous solid. A further quantity (0.20 g.) of neutral sugars was extracted from this with dry ether and added to (T) above. The residue consisted of barium uronates (U) (2.10g.).

Fractionation of the Neutral Methylated Sugars (T)

The mixture of neutral methylated sugars (1.74 g.) was fractionated by partition chromatography on a column of cellulose (850 x 30 mm.) using as eluants light petroleum (70 parts): n-butanol (30 parts) saturated with water, changing by stages to light petroleum (60 parts):n-butanol (40 parts) saturated with water and finally to water. (7-9 ml. fractions were collected).

Fraction	Solvent petrol: n-butanol	Tubes	Colour of Spot	R_G	Weight after purifi-:cation.
T_1	70:30	221-240	Red brown	0.88	0.127 g.
T_2	70:30	241-290	Red brown with yell-:ow brown tail.	0.86	0.307 g.
T_3	60:40	620-730	Red brown	0.71	0.435 g.
T_4	60:40	1350-1480	Yellow brown	0.55	0.140 g.
T_5	Water				0.240 g.
			Total		1.249 g.

R_G values were taken in solvent III.

Fraction T_1: 0.127 g. $[\alpha]_D^{18}$ + 113° (c. 1.14 in H_2O)
R_G 0.88; OMe 51.9%.

(calc. for a tetra-O-methylhexose: OMe 52.5%)

These results indicated 2:3:4:6-tetra-O-:methyl-D-galactose which has $[\alpha]_D$ + 113° (111); R_G 0.88. The sugar was characterised by heating a portion (0.076 g.) under reflux with aniline (0.040 ml.) in ethanol (4 ml.) Distillation of the solvent left needles (0.054g.) which after recrystallisation from anhydrous ethanol had m.p. and mixed m.p. 190-191° and $[\alpha]_D^{18}$ -69°→ +35° (equilibrium value)(c. 0.75 in acetone); 2:3:4:6-tetra-O-methyl-N-phenyl-D-galactosylamine has $[\alpha]_D$ -77°→+37° (111).

Fraction T_2: 0.307 g.

That this fraction comprised two components was apparent from the following evidence.

(i) Separation into two spots was achieved in solvent system III saturated with boric acid or in solvent VI, the latter being preferable.

(ii) Electroionophoresis showed two components of M_G 0.35 and 0.0 respectively.

Resolution of T_2

The mixture was separated by partition chromatography on a column of "Celite 535": the Celite, which had been treated with concentrated hydrochloric acid for 18 hours at room temperature, washed free from acid and dried at 110° was damped with the aqueous phase of solvent VI (1ml. per g. of Celite). After conversion to a slurry with the organic phase, the celite was poured into a glass column and allowed to settle, air bubbles being worked out by stirring. The column was then compressed to about two-thirds of its original length (final dimensions 500 x 30 m.m.) The syrup (T_2) (0.305 g.) was dissolved in the organic phase of solvent VI (3 ml.) and absorbed on dry Celite (3 g.). The mixture was packed on top of the prepared column and developed with the

organic phase of the solvent, the eluant being fractionated in 25 ml. portions. Though kept at constant temperature the column was not in equili-:brium since water was deposited from the eluate on standing; the separation was not, however, significantly affected.

T_{2A} Tubes 31-41 A syrup 0.214 g.; $[\alpha]_D^{18}$ + 112° (c., 2.14 in H_2O) R_G 0.88; OMe 52.0%

This material was added to fraction T_1.

T_{2B} Tube 51. 0.036 g.; $[\alpha]_D^{18}$ + 23° (c. 2.3 in H_2O); R_G 0.88; OMe 32.9% (calc. for a di-O-:methyl-deoxyhexose, OMe 32.3%) M_G 0.35.

On standing in vacuo over phosphorus pentoxide the material began to crystallise giving needles, m.p. 93-94°. Demethylation gave rhamnose (paper chromatography). This evidence identified the fraction as 3:4-di-O-:methyl-L-rhamnose which has $[\alpha]_D$ + 18.2°; m.p. 94-95° (112) and R_G 0.875; M_G 0.36 (113).

Fraction T_3 A syrup 0.435 g.; $[\alpha]_D^{20}$ + 96° (c. 1.77 in H_2O) R_G 0.71; OMe 40.9% (calc. for a tri-O-methylhexose, OMe 41.9%)

These results indicated 2:3:6-tri-O-methyl-D-galactose which has $[\alpha]_D$ + 87° (114); R_G 0.71. A portion (0.090 g.) was oxidised with bromine water for 4 days at room temperature, aerated to remove excess of bromine and neutralised with

silver carbonate. After filtration, silver was removed with hydrogen sulphide and the solution was again filtered then evaporated to dryness. The residue was extracted with ethyl acetate and the extracts evaporated to a syrup (0.063 g.) which crystallised. Recrystallisation from benzene-light petroleum gave needles having m.p. and mixed m.p. 97-99° and $[\alpha]_D^{18}$ - 40 → - 30° (equilibrium value) (c., 1.15 in H_2O); 2:3:6-:tri-O-methyl-D-galactonolactone has $[\alpha]_D$ - 40 → - 28° (115) Chromatographic examination of the crude lactone (solvents IV and V) showed only one component which was identical with 2:3:6-tri-O-methyl-D-galactono-:lactone (detected by spraying with alkaline hydroxylamine hydrochloride, then ferric chloride solutions (116)).

Fraction T_4 Crystals 0.140 g.; m.p. and mixed m.p. with 3-O-methyl-L-rhamnose 114-115°; $[\alpha]_D^{18}$ + 30°; (c. 0.96 in H_2O); R_G 0.55; OMe 17.0% (calc. for a mono-O-methyl-deoxyhexose, OMe 17.4%)

These results indicated 3-O-methyl-L-:rhamnose which has $[\alpha]_D$ + 35° (117); R_G 0.55. The X-ray powder photograph was identical with that of the standard and distinct from that of 4-O-methyl-L-rhamnose. A portion (0.052 g.)

was oxidized with bromine water and worked up in the same way as fraction T_3. The clear syrup (.0093 g.) obtained after distillation under high vacuum (bath temp. 150-160°/0.02 mm.) had $[\alpha]_D^{15}$ - 20° (equilibrium value (c. 0.93 in H_2O). Recorded value for 3-O-methyl-L-rhamnonolactone is $[\alpha]_D$ - 20° → - 18° (110).

Fraction T_5

This fraction gave no indication of neutral sugars (paper chromatography) and was added to the barium uronates (U) (page 94).

Examination of the Barium Uronates (U) from the hydrolysed methylated Polysaccharide.

The barium uronates (2.34 g.) were dissolved in water and barium was removed with ion-exchange resin (Amberlite IR-120,H). On evaporation the free uronic acids were obtained as a red syrup (V) (1.70 g.) which resisted fractionation on "Celite" with solvent IV. Fractionation was achieved, however, with the solvent system n-butanol:n-butyl acetate:acetic acid:water (50:6:4:40) using a method analogous to that for the resolution of fraction T_2 (page 96). Fractions of 25 ml. volume were taken.

Fractions obtained:

Fraction V_1 Tubes 14-26, 1.00 g.; $[\alpha]_D^{18}$ + 59° (c. 0.93 in H_2O);

Chromatography in an acidic solvent (solvent IV) showed this fraction to be identical with 2:3:4-tri-O-methyl-D-glucuronic acid. In methylethyl ketone:water:ammonia (page 101) a spot corresponding to dimethyl rhamnose was also detected.

Reduction, hydrolysis and fractionation of V_1

A portion (0.350 g.) was refluxed for 6 hours with methanolic hydrogen chloride (3%; 40 ml.). The solution was neutralised with ethereal diazomethane and immediately evaporated to dryness. The product was dissolved in dry tetrahydrofuran (5 ml.) and a saturated solution of lithium aluminium hydride in dry tetrahydrofuran was added dropwise. The solution was refluxed gently for 30 minutes, cooled and excess of lithium aluminium hydride destroyed by cautious addition of ethyl acetate. Water was added, the solution was evaporated to dryness and the residues were extracted with

boiling acetone, then dry chloroform. On evap-:oration of the extracts a syrup was obtained which was hydrolysed with N-hydrochloric acid (5 ml.) at 100° for 6 hours, neutralised with silver carbonate and filtered. The filtrate was treated with hydrogen sulphide, evaporated to dryness and the residues extracted with dry acetone. Removal of the acetone gave a syrup (W) (0.279 g.). The syrup (W) was partitioned on a column of cellulose (25 x 420mm.) using methyl ethyl ketone half-saturated with water and containing 1% of concentrated ammonia as eluant. Tubes for chromatographic inspection were evaporated at 30-40° in a stream of nitrogen.

Fraction W_1 0.054 g.

This fraction was further purified on thick paper. A syrup (0.028 g.) $[\alpha]_D^{18}$ + 17° (c. 0.36 in H_2O); R_G 0.88; M_G 0.36; OMe 29.5% (calc. for a di-O-methyl hexose, OMe 32.3%) was finally isolated. On demethylation, rhamnose was produced. The syrup was oxidized on standing in aqueous solution with potassium periodate(132).

Overlap fraction: 0.137 g.; $[\alpha]_D^{18}$ + 47° Paper chromatography showed the presence of fractions W_1 and W_2.

Fraction W_2 0.062 g. $[\alpha]_D^{18}$ + 65° (c. 0.18 in H_2O); R_G 0.85; OMe 41.3% (calc. for a tri-O-methylhexose, OMe 41.9%)

These results indicated 2:3:4-tri-O-methyl-:D-glucose which has $[\alpha]_D$ + 60° (118), R_G 0.85. A portion (0.048 g.) was heated under reflux with aniline (0.030 g.) and ethanol (3 ml.) in an atmosphere of carbon dioxide for 3 hours. The solution was evaporated in a stream of nitrogen at 40° to a syrupy residue which was moistened with a few drops of ethanol. Ether (2 ml.) was added, and the brown precipitate removed by filtration. Light petroleum (60-80°) was then added and the solution was allowed to stand, when crystallisation occurred. After recrystallisation from ethanol-ether-light petroleum, needles were obtained having m.p. and mixed m.p. 141-143°. 2:3:4-tri-O-methyl-:N-phenyl-D-glucosylamine has m.p. 145-146° (118).

Fraction V_2 Tubes 34-64; 0.239 g.

This fraction, which was contaminated with neutral sugars (mainly 3-O-methyl-L-rhamnose; paper chromatography) was purified by elution on thick paper with methylethyl ketone:water:ammonia. The uronic acid remained on the starting-line and was then eluted with water,

deionised (Amberlite IR-120, H) and evaporated to a syrup (0.083 g.) $[\alpha]_D^{18}$ + 80° (c. 0.33 in H_2O). A portion (0.060 g.) was converted to the ester glycoside, reduced (lithium aluminium hydride) and hydrolysed. The resulting syrup contained four components (paper chromatography):

R_G	Colour (aniline oxalate spray).	Possible Identity
0.85	Red-brown	2:3:4-Tri-O-methyl-D-:glucose.
0.56	Lemon-brown	3-O-Methyl-L-rhamnose
0.28	Brown	Rhamnose
0.23	Red-brown	Mono-O-methylhexose.

Fraction V_3 Tubes 64-84; 0.100 g.; $[\alpha]_D^{20}$ + 93° (c. 0.38 in H_2O)

This fraction was free from neutral sugars (paper chromatography); it was converted to the methyl ester, methyl glycoside, reduced with lithium aluminium hydride and hydrolysed. The following ~~seven~~ six components were found in the product (paper chromatography):

R_G	Colour of Spot	Possible Identity
0.85	Red-brown	2:3:4-Tri-O-methyl-D-:glucose
0.71	Red-brown	2:3:6-Tri-O-methyl-D-:galactose
0.56	Lemon-brown	3-O-Methyl-L-rhamnose
0.46	Red-brown	Di-O-methylhexose.
0.28	Brown	Rhamnose
0.23	Red-brown	Mono-O-methylhexose

Reduction of the Methylated Polysaccharide

The methylated gum (0.75 g.) was dissolved in dry tetrahydrofuran (15 ml.) and added slowly and with stirring to a saturated solution of lithium aluminium hydride in dry tetrahydrofuran (3 ml.). The solution was refluxed for 30 minutes, then allowed to stand overnight. Excess of lithium aluminium hydride was destroyed by addition of ethyl acetate, then the solution was acidified with dilute sulphuric acid and extracted with chloroform. The extracts were washed with saturated sodium bicarbonate solution, dried over sodium sulphate and the solvent removed by distillation, leaving a solid (0.610 g.) having $[\alpha]_D^{18}$ + 75°. (c. 1.03 in $CHCl_3$).

Remethylation and Hydrolysis of the Reduced Methylated Polysaccharide.

The reduced methylated polysaccharide (0.41g.)

was treated four times with methyl iodide and silver oxide to yield the remethylated reduced methylated polysaccharide (0.310g.) having methoxyl content of 41.8% and $[\alpha]_D^{18}$ + 45.8° (c. 2.58 in $CHCl_3$). After methanolysis with hydrogen chloride (4%) in methanol for 8 hours under reflux, the product was hydrolysed with N-hydrochloric acid for 16 hours. The solution was neutralised with silver carbonate, filtered, then refiltered after passage of hydrogen sulphide. The solution was taken to dryness and the residues extracted with dry acetone. Removal of acetone gave a syrup (0.238 g.) containing methylated sugars. Chromatography of the hydrolysate (0.208 g.) (solvent III) showed the following components:

R_G	Possible Identity
1.00	2:3:4:6-Tetra-O-methyl-D-glucose
0.85	2:3:4:6-tetra-O-methyl-D-galactose + 3:4-Di-O-methyl-L-rhamnose.
0.71	2:3:6-Tri-O-methyl-D-galactose
0.57	3-O-Methyl-L-rhamnose
0.46) 0.42)	Di-O-methylhexose
0.28	Rhamnose
0.23	Mono-O-methylhexose

Attempted Fractionation of the Uronic Acids on a Column of Ion-exchange Resin

A column of anion-exchange resin (Amberlite IRA-400, acetate form) was prepared according to Matheson (110) and a portion of the uronic acids (M-page 79) (3.39 g.) absorbed on it. Elution was carried out with increasing concentrations of aqueous acetic acid after removal of neutral sugars (0.074 g.) by washing with water. The eluate was fractionated and those of similar chromatographic composition were bulked together and evaporated to dryness cautiously with addition of portions of water when small volume was reached to avoid undue concentration of acetic acid in the solutions. Contamination of the eluate was caused by the slight solubility of the resin; this was best removed by intensive drying of the fractions and aqueous extraction. Only two pure fractions were obtained:

One (M_1) (0.008g.) eluted with 2-3% acetic acid, was chromatographically identical with glucurone.

The other (M_2) (0.355 g.) eluted with 3.5-5% acetic acid, had $[\alpha]_D^{18}$ + 63° (c. 1.51 in H_2O) R_{Gal} 0.24 M_G 0.72. The equivalent weight (by direct titration with sodium hydroxide in presence of methyl red) was 332. An aldo-

:biuronic acid composed of rhamnose and glucuronic acid would have an equivalent weight of 340. Determination of iodine uptake in alkali gave results which were not reproducible; rhamnose was found to behave similarly. Complete hydro-:lysis of M_2 (2N-sulphuric acid at 100° for 24 hours) gave rhamnose and glucuronic acid (paper chromatography). Partial hydrolysis (N-sulphuric acid at 100° for 4 hours) gave the same two compon-:ents and unhydrolysed material. A second portion of M_2 was submitted to reduction with aqueous sodium borohydride solution followed by hydrolysis (1.5 N-sulphuric acid at 100° for 6 hours) and neutralisation with barium carbonate. After deionisation (Amberlite IR-120, H), chromatography showed only glucuronic acid, neutral sugars being absent.

The remainder of the material (0.320g.) was methylated three times at room temperature with sodium hydroxide (40%; 7.5 ml.) and dimethyl sulphate (3.0 ml.) in an atmosphere of nitrogen then further methylated by two additions one at 50° and the other at 70°. After acidification, the solution was extracted with chloroform at room temperature, the extracts being washed with

sodium bicarbonate, dried over sodium sulphate and evaporated. The material obtained (0.148 g.) was further methylated with methyl iodide (2.3 ml.) and silver oxide (1.2 g.). The syrup isolated (0.140 g.) was dissolved in dry methylal (2.0 ml.) and reduced by addition of lithium aluminium hydride (0.1 g.) in the same solvent (1 ml.). Excess of reagent was destroyed by cautious addition of water and the product (0.098 g.) isolated by methylal extraction. After two further methylations with methyl iodide and silver oxide, a syrup was obtained (0.088 g.) which was hydrolysed (N-sulphuric acid at 100° for 6 hours) yielding after neutralisation a syrup (0.060 g.) which was separated on thick paper (Whatman 3 M.M. solvent III).

The first component was identical with 2:3:4:6-tetra-O-methyl glucose chromatographically and electroionophoretically. Demethylation gave glucose.

The second component was identical with 3:4-di-O-methyl rhamnose chromatographically and electroionophoretically (M_G 0.36).

The third component, which was present in very small quantity, had R_G 0.76 and gave

glucose on demethylation.

The other fractions obtained from the resin column were impure; their compositions are given below:

Acetic Acid Concentration	Weight (g.)	R_{Gal}	Hydrolysis Products
0.1-0.3%	0.160	0.27 0.39 0.90 1.05	Rh, Ga, Glu
0.7-1.0%	0.238	1.11 0.27 faint 0.39 "	Rh, Glu
1.25-2.0%	0.240	1.11 faint 0.98	Rh, Ga (trace), Glu
5.0-12.5%	0.080	1.01 0.37 0.14	Rh, Ga, Glu.

Key: Rh:- Rhamnose; Ga:- Galactose
Glu:- Glucuronic acid.

BIBLIOGRAPHY

(1) Jones and Smith, Advances in Carbohydrate Chemistry, Academic Press, New York, 1949, 4, 243.

(2) Anderson and Sands, ibid. 1945, 1, 329.

(3) Hirst and Jones, J. Soc. Dyers and Colour-:ists, 1947, 63, 249.

(4) Hirst, Endeavour, 1951, 10, 106.

(5) Whistler and Smart, Polysaccharide Chemistry, Academic Press, New York, 1953, 304.

(6) Hirst and Jones, Research, 1951, 4, 411.

(7) Hirst and Jones, Modern Methods of Plant Analysis, Springer-:Verlag, Berlin, 1955, 2, 275.

(8) Heyne and Whist-:ler, J. Amer. Chem. Soc., 1948, 70, 2249.

(9) Hirst and Jones J., 1948, 1278.

(10) Lythgoe and Trippett, J., 1950, 1983.

(11) Charlson, Nunn and Stephen, J., 1955, 1428.

(12) Whistler and Conrad, J. Amer. Chem. Soc., 1954 76, 3544.

(13) Andrews, Ball and Jones, J., 1953, 4090.

(14) Andrews and Jones J., 1954, 4134.

(15) Andrews and Jones J., 1955, 583.

(16) Charlson, Nunn and Stephen, J., 1955, 269.

(17) Hirst and Perlin J., 1954, 2622.

(18) Brown, Hirst and Jones J., 1948, 1677.

(19) Smith, J., 1939, 744.

(20) Stephen, J., 1951, 646.

(21) Jones, J., 1939, 558.

(22) Brown, Hirst and Jones, J., 1949, 1761.

(23) Hirst and Jones J., 1938, 1174.

(24) Hirst and Jones J., 1947, 1064.

(25) Jones and Nunn, J. Amer. Chem. Soc., 1955, 77, 5745.

(26) Aspinall, Hirst and Wickstrøm, J., 1955, 1160.

(27) Connell, Hainsworth Hirst and Jones. J., 1950, 1696.

(28) Stephen, J., 1956, 4487.

(29) Hirst and Dunstan J., 1953, 2332.

(30) Aspinall, Hirst and Matheson, J., 1956, 989.

(31) White, J. Amer. Chem. Soc., 1947, 69, 2264.

(32) Parikh, Ingle and Bhide, J. Indian Chem. Soc., 1956, 33, 119.

(33) Hough, Jones and Wadman, J., 1952, 796.

(34) Mukherjee and Scrivastava, J. Amer. Chem. Soc. 1955, 77, 422.

(35) El-Khadem and Megahed, J., 1956, 3953.

(36) Jones, J., 1950, 534.

(37) McIlroy, J., 1951, 1918.

(38) Anderson and Ledbetter, J. Amer. Pharm. Assoc., 1951, 40, 623.

(39) Anderson and Harris, J. Amer. Pharm. Assoc., 1952, 41, 529.

(40) Hirst, Hough and Jones, J., 1949, 3145.

(41) Hough and Jones J., 1950, 1199.

(42) Jones, J., 1949, 3141.

(43) Bell and Young, Biochem. J., 1934, 28, 882.

(44) Thomas and Murray, J. Phys. Chem., 1928, 32, 676.

(45) Harris-Seybold Co., Brit. 689, 623. Chem. Abs., 1953, 47, 10256.

(46) Parikh, Ingle and Bhide, J. Indian Chem. Soc., 1956, 33, 125.

(47) Heidelberger, Adams and Dische, J. Amer. Chem. Soc., 1956, 78, 2853.

(48) Walder, Food, 1948, 17, 356, 360.
ibid, 1949, 18, 4.

(49) White, J. Amer. Chem. Soc., 1947, 69, 715.

(50) Cramer, J. Franklin Inst., 1953, 256, 93.

(51) Hudson, J. Amer. Chem. Soc., 1951, 73, 4038.

(52) Weinmann, Ber., 1929, 62, 1637.

(53) Joubert, J.S. African Chem. Inst., 1954, 7, 107:
Chem. Abs., 1955, 49, 8622

(54) Jones and Pridham, Modern Methods of Plant Analysis, Springer-Verlag, Berlin, 1955, 2, 282.

(55) Colvin, Cook and Adams, Canad. J. Chem., 1952, 30, 603.

(56) Selby, J., 1953, 2504.

(57) Clark, Ind. Eng. Chem. Anal. 1936, 8, 487.
ibid, 1937, 9, 539.

(58) Baker and Hulton, Biochem. J., 1920, 14, 754.

(59) Tracey, Biochem. J., 1948, 43, 185.

(60) McCready, Swenson and Maclay, Ind. Eng. Chem. Anal., 1946, 18, 290.

(61) Partridge, Nature, 1946, 158, 270.

(62) Partridge and Westall, Biochem. J., 1948, 42, 238.

(63) Jermyn and Isherwood, Biochem. J., 1949, 44, 402.

(64) Hirst and Jones Discuss. Faraday Soc., 1949, 7, 271.

(65) Barker and Smith Chem. and Ind., 1954, 32 19.

(66) Hirst, J., 1942, 70.

(67) Edington and Percival, J., 1955, 3554.

(68) Smith and Spriestersbach, Nature, 1954, 174, 466.

(69) Trevelyan, Procter and Harrison, Nature, 1950, 166, 444.

(70) Lemieux and Bauer Analyt. Chem., 1954, 26, 920.

(71) Hough, Jones and Wadman, J., 1950, 1702.

(72) McNair Scott and Cohen, J. Biol. Chem., 1951, 188, 509.

Ayres, Dingle, Phipps, Reid and Solomons, Nature, 1952, 170, 834.

(73) Flood, Hirst and Jones, J., 1948, 1679.

(74) Hirst and Jones, J., 1949, 1659.

(75) Pridham, Analyt. Chem., 1956, 28, 1967.

(76) Whistler and Durso, J. Amer. Chem. Soc., 1950, 72, 677.

(77) Hough, Jones and Wadman, J., 1949, 2511.

(78) Lemieux, Bishop and Pelletier, Canad. J. Chem., 1956, 34, 1365.

(79) Gardell, Acta. Chem. Scand., 1953, 7, 201.

(80) Khym and Zill, J. Amer. Chem. Soc., 1951, 73, 2399.
ibid, 1952, 74, 2090

(81) Haworth, J., 1915, 107, 8.

(82) Purdie and Irvine, J., 1903, 83, 1021.

(83) Fear and Menzies, J., 1926, 937.

(84) Kuhn and Baer, Ber., 1955, 88, 1537.
idem ibid, 1956, 89, 504.
Kuhn, Low and Trischmann, ibid, 1955, 88, 1492.
idem Angew. Chem., 1955, 67, 32.

(85) Foster, Chem. and Ind., 1952, 1050.

(86) Bell, J., 1944, 473.
Bell and Palmer, Nature, 1949, 163, 846.

(87) White, J. Amer. Chem. Soc.,
1952, 74, 3966.
1953, 75, 4692.
1954, 76, 4906.

(88) Whelan and Morgan, Chem. and Ind., 1954, 78.

(89) Whistler and Smart Polysaccharide Chemistry, Academic Press, New York, 1953, 317.

(90) Challinor, Haworth and Hirst, J., 1931, 258.

(91) Cuneen and Smith, J., 1948, 1141.

(92) Abdel-Akher, Smith and Spriestersbach J., 1952, 3637.

(93) Unruh, McGee, Fowler and Kenyon, J. Amer. Chem. Soc., 1947, 69, 349.

(94) Dutton and Smith, J. Amer. Chem. Soc., 1956, 78, 2505.

(95) McDonald, Ionography, Year Book Publishers, Chicago, 1955, 136.

(96) Dische and Shettles, J. Biol. Chem., 1948, 175, 595.

(97) Butler and Cretcher J. Amer. Chem. Soc., 1931, 53, 4160.

(98) Jones and Pridham, Biochem. J., 1954, 58, 288.

(99) Ingles and Israel, J., 1948, 810.

(100) James and Smith, J., 1945, 739, 746, 749.

(101) Wolfrom, J. Amer. Chem. Soc., 1930, 52, 2466.

(102) Beauquesne, Compt. rend., 1946, 222, 1056.

(103) Freudenberg, Plankenhorn and Boppel, Ber., 1938, 71, 2435.

(104) Rao and Sharma, Proc. Indian Acad. Sci., 1957, 45, 24.

(105) Ehrlich, Ber., 1932, 65, 352.

(106) Keilin and Hartree, Biochem. J., 1948, 42, 230.

(107) Shaffer and Somogyi J. Biol. Chem., 1933, 100 695.

Hanes and Cattle, Proc. Roy. Soc., 1938, B.125, 387.

(108) Manners, Unpublished work.

(109) Nelson, J. Biol. Chem., 1944, 153, 375.

(110) Matheson, Ph. D. Thesis, Edinburgh 1955.

(111) Haworth, Loach and Long, J., 1927, 3146.

(112) Tipson, Christmann and Levene, J. Biol. Chem., 1939, 128, 609.

(113) Foster, Chem. and Ind., 1952, 828.

(114) Haworth, Raistrick and Stacey, Biochem. J., 1937, 31, 640.

(115) Unpublished work, quoted by Charlson, Nunn and Stephen, J., 1955, 1428.

(116) Abdel-Akher and Smith, J. Amer. Chem. Soc., 1951, 73, 5859.

(117) Gorrod and Jones, J., 1954, 2522.

(118) Peat, Schluchterer and Stacey, J., 1939, 581.

(119) Anderson and Blake, J. Amer. Pharm. Assoc., 1953, 42, 662.

(120) Eisman, Cooper and Jaconia, J. Amer. Pharm. Assoc., 1957, 46, 144.

(121) Mukherjee and Banerjee, J. Indian Chem. Soc., 1948, 25, 63.

(122) Mathur and Mukherjee J. Sci. Ind. Res., India, 1952, 11B, 544: Chem. Abs. 1953, 47, 10881.

(123) Hamilton, Spriestersbach and Smith, J. Amer. Chem. Soc., 1957, 79, 443.

(124) Brown, Hirst and Jones, J., 1949, 1757.

(125) Stephen, J., 1957, 1919.

(126) Smith, Personal communication.

(127) Hirst and Jones, J., 1946, 506.

(128) Smith, J., 1940, 1035.

(129) White, J. Amer. Chem. Soc., 1946, 68, 272.

(130) Jones, J., 1947, 1055.

(131) Schlubach Annalen, 1955, 595, 236.

(132) Butler, Lloyd and Stacey J., 1955, 1531.

ACKNOWLEDGMENTS

The author wishes to express his sincere thanks to Professor E. L. Hirst and to Dr. E. E. Percival for their advice and guidance throughout the course of this work, and to the Department of Scientific and Industrial Research for a maintenance grant. Thanks are also due to Dr. C. T. Greenwood for the preparation of the Schlieren diagram, to Dr. D. J. Manners for guidance in the enzymic work and to the staff of the Crystallography laboratory for the preparation of the X-ray powder photograph.